# Role of Sucrose or STS Pulsing in the Regulation of Cut Flower Senescence of *Gentiana triflora*

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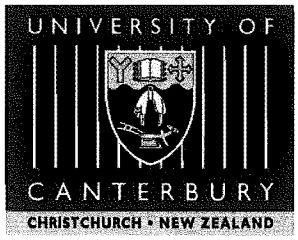


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# Abstract

A controlled rather than an unreliable spontaneous *in vitro* flowering system of *Gentiana triflora* was established successfully. *In vitro* flower of gentian is of great similarity to *in vivo* flower, lending support to the former as an alternative in the proposed study on gentian flower physiology. The flowers can be obtained from more than 80% of the *in vitro*-cultured nodal explants in 12 weeks. Pulsing with 87.6 mM sucrose or 0.5 mM STS solution clearly improved bud opening and flower vase life in cut gentian. Total soluble sugar concentration increased markedly and starch content was obviously low in the petals after the sucrose treatment and this was confirmed using radioactive $^{14}$C-sucrose pulsing treatment. Exogenous ethylene could induce autocatalytic ethylene production in gentian petal and resulted in shortened flower vase life, while ethylene production of the flowers could be suppressed by the sucrose or STS treatment. Amylase activities were significantly higher in 3% sucrose (w/v) or 0.5 mM STS treated flowers than in water treated control samples. Gentian flower senescence was associated with reduction of the protein content in the petals. Sucrose or STS pulsing treatment could increase protein content in the petals in comparison to that of water treated sample. A new polypeptide of low molecular weight was detected on SDS-PAGE gels used for analysis of the protein profiles of the petals treated with sucrose or STS. This suggested the possible changes in gene expression resulting from the pulsing treatments. Finally, a 931-bp cDNA fragment was obtained using a pair of primers designed from the conserved region of ACC oxidase gene sequences from several flowers using RT-PCR technique. Dot blot data suggested that ACC oxidase gene expression in the petal of cut gentian flowers could be suppressed by the administration of sucrose or STS. This indicated that changes in petal physiology in response to sucrose or STS were associated with ethylene-induced alterations in gene expression. Both sucrose and STS could be inhibitors of ethylene biosynthesis via regulating ACC oxidase gene expression.

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# List of Abbreviations

| | |
|---|---|
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| ANOVA | analysis of variance |
| BA | 6-benzyladenine |
| Bq | Becquerel (Radioactive disintegration) |
| CE | crude enzyme |
| cDNA | complimentary deoxyribose nucleic acid |
| DEPC | diethyl pyrocarbonate |
| $dH_2O$ | distilled water |
| DIG | digoxigenin |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribose nucleic acid |
| dUTP | 2'-deoxy-uridine-5'-triphosphate |
| EDTA | ethylenediaminetetraacetic acid (sodium salt) |
| $GA_3$ | gibberellic acid |
| HCl | hydrochloride acid |
| HQS | 8-hydroxyquinoline |
| IEF | isoelectric focusing |
| PAGE | polyacrylamide gel electrophoresis |
| PC | paper chromatography |
| PCR | polymerase chain reaction |
| PPFD | photosynthetic photon flux density |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RT-PCR | reverse transcription - polymerase chain reaction |
| SDS | sodium dodecyl sulphate |

| | |
|---|---|
| SEM | Scanning electron microscope |
| STS | silver thiosulphate |
| TEMED | N,N,N',N'-tetramethylethylenedimine |
| Tris | tris (hydroxymethyl) aminomethane |
| UV | ultraviolet |
| WPM | Woody Plant Medium |

# Chapter 1. Introduction

## 1.1. Introduction to Gentians

### 1.1.1. *The History of Gentians*

Gentians are characteristic of the flora in alpine regions. Their name is derived from Gentius, a king of Illyria in the Adriatic in the second century AD, who is always credited with having given his name to the family (Wagner, 1994). A more romantic story is connected with Ladishaus, King of Hungary (1440-1457). He prayed that if he shot an arrow in the air the Almighty should guide it to some herb that would relieve the suffering of his plague-stricken people — and it landed on a gentian.

According to Bartlett (1981), by the sixteenth century the wonderful plant had been introduced to England and Robert Turner records that 'it resists poisons, putrefaction, and the pestilence and helps digestion'. In Switzerland, it was used as a cure for those who were bewitched and 'for those that had taken somewhat to fall in love with another body'. As an antidote to love *G. lutea* may have had some effect. Certainly the acrid essence distilled from its root is quite a knockout. The strong yellow drink Suze, which is popular in France and Switzerland, was originally made from it. The bitter spirit of gentians has been used instead of hops in Swiss beer, and a distillation from the marsh gentian *G. pneumonanthe* was once used, as its name suggests, as a curative for lung diseases.

In the present century gardeners have shown growing interest in gentians. The creation of natural alpine gardens within botanical gardens demonstrated the breadth and variety of the genus to a wider public, and led to the construction of numerous private rock gardens. Further interest was stimulated from the discovery of the Chinese autumn-flowering species by British plant hunters. Their introduction made it possible for gardeners to have gentians in flower continuously from early spring to late autumn. These new species brought completely novel shades of blue into the garden, including pure ultramarine blue and turquoise ones which are almost unknown in any other flower. Although most gentian flowers are blue, other colours such as violet, scarlet, pink, white, yellow, red and even green flowers are known to exist (Kohlein, 1991).

Over the last decade, a range of gentian cultivars has been cultivated as cut flowers; the majority originated from Japan (Nelson, 1995). Gentians have become established as a new cut flower crop in the world market.

### 1.1.2. *The Distribution of Gentians*

Gentians occur widely throughout the world. In North America they extend in the north from Alaska to Canada and the United States. Leaving the United States, they extend through Mexico and ultimately into South America. In South America, on both sides of the Equator, certain species are found in Colombia, Venezuela, Ecuador and Peru. Further south they are found in Chile, Bolivia, and Argentina as far as the Magellan Region. Throughout Europe more gentian species are found in nearly every country, even as far north as Nova Zembla; and right through the continent into Asia. The continent of Asia has a greater number of gentian species than the other continents (Wilkie, 1950). Their distribution extends through the northern part, from the Ural Mountains in the west to Kamcharka in the east, and in a southerly direction through Mongolia, Japan and China. India is also rich in gentians. Throughout Nepal, Bhutan and Burma there is a wonderful range of species. From there gentians are found throughout Siam, the Philippine Islands, and Malaya, and at the Equator they are found in Sumatra, Borneo, Celebes Island, Java and New Guinea. Continuing into Australia

and New Zealand, their distribution extends as far south as the Auckland Islands and Campbell Islands (Sorensen, 1951). Species have also been recorded from South Africa, from Sierra Leone and Morocco (Wilkie, 1950). Only the Middle East has no native gentian species (Kohlein, 1991).

### 1.1.3. *The Classification of Gentians*

Gentian is the common name of the genus *Gentiana* that belongs to the family Gentianaceae. It is of great interest both to botanists and laymen because of its large diversity and the beauty of the flowers of some species. Nelson (1995) indicated that there are 400 species in the genus. Only a few of these species are of economic importance. There is a range of cultivars of Gentians used for cut flower production, the majority of which were sourced from Japan (Nelson, 1995).

According to Pringle (1978), the classification of the genus *Gentiana* began relatively early and goes back to the first names in Reneaulme's *Specimen Historiae Plantarum* in 1611 (Kohlein, 1991). Further attempts appeared in Linnaeus' *Species Plantarum* in 1753, Necker's *Elementa Botanica* in 1790 and Froelich's *De Gentiana Libellus Sistens* in 1796. Froelich's classification was accepted, with minor modifications, by Lamarck and de Candolle in 1805 and used in the *Flora Francaise*. This was the first work in which the genus was subdivided into sections in the present sense of the word. Another writer who studied the question was Link (*Enumeratio* 1821-1822 and *Hortus Regius* 1829-1833). Dumortier divided the genus into five sections in this *Florula Belgica* in 1827. Further breakdown into ten subdivisions followed in Gaudin's *Flora Helvetica* (1828-1833). Bunge's monograph on the gentian in 1829, Grisebach's review of Gentianaceae in Hooker's *Flora Boreali-Americanae* and Grisebach's two major treatises on Gentianaceae (1838 and 1845) resulted in an overall classification which approached more closely to modern ideas. The most up-to-date classification of the entire genus is the one undertaken by Kusnezow. His monograph entitled *Subgenus Eugentiana* in 1895 and his chapter on gentians in 'Die naturliche Pflanzenfamilien' in 1895 is today still the basis of the taxonomy and classification of

the genus. However, in addition there are numerous new species discovered since Kusnezow's time.

The gentian family is Gentianaceae of the order Gentianales, class Magnoliopsida, and division Magnoliophyta. The representative genus is *Gentiana* L (Crosby, 1995). The main species used in this study is *Gentiana triflora* Pall, subspecies *Gentiana axillariflora*, cultivar 'Akita Blue'. Figure 1-1 shows the flowers of *G. triflora*.

*Gentiana triflora* Pall. var. *axilariflora* Akita Blue

**Figure 1-1** ***Gentiana triflora* Pall. var. *axillariflora***

The flowers are pure ultramarine blue, and grown for the cut-flower trade. The form shown here has the majority of its flowers in the terminal cluster.

The stem of cultivated *G. triflora* is grown to 100 - 200 cm high. The leaves are arranged in pairs, each pair being at right angles to the next and not forming a tube round the stem. The leaves at the base of the stem are smaller than those at the top. The flowers are carried in the upper leaf axils and at the top of the stem. The terminal flowers are usually grouped, while those in the leaf axils are single and have a short stalk. The bell-shaped corolla is over 3 cm long, with rounded, ovate, erect lobes. The flowers are ocean blue, and generally open in August to October in Northern Hemisphere and January to March in Southern Hemisphere.

### 1.1.4. *Production of gentians in New Zealand*

Approximately half ($NZ 50 million) of the total New Zealand cut flower production is exported every year. This figure is increasing again after a dip in 1996 and 1997 during the Asian economic crisis. This constitutes an international market share of around 0.5 percent. New Zealand's reliance on Japan as an export destination is diminishing (Statistics New Zealand 2000). Almost 70% of exported flowers are currently sold to Japan, but diversification into other markets such as the United States, Europe, and other Asian countries is occurring (Statistics New Zealand 2000). New Zealand flower exports earned about $NZ 60 million in 1996, and the industry is planning to increase that figure to $NZ 80 million by the year 2000 (Statistics New Zealand 2000).

Among the cut flowers exported the newly developed cut flower crops are more preferable to the traditional species in the world markets. Table 1-1 shows the export values of few important cut flower species basing on the latest statistical data (Statistics New Zealand 1998). It appears that not only the values of new cut flower species (Gentians and Sandersonia) increased comparing with the traditional cut flowers, but also the value in Gentians increased more comparing with any other new or traditional cut flowers listed in the table.

Ornamental gentians are native of eastern Siberia, Korea, Sakhalin and Japan (Kohlein, 1991). Some new hybrids have been raised for cut flower or pot plant production in

recent years. It has been introduced into New Zealand from Japan since early 1990's and became a new summer cut flower in New Zealand. New cultivars are being introduced continuously each year. They are grown in varying locations throughout New Zealand from South Auckland, Taupo and Palmerston North to Canterbury, Otago and Southland (Nelson, 1995). In New Zealand, the production of gentians has been expanded for export beginning in 1993. Table 1-2 shows gentian export values from 1993 to 1998.

**Table 1-1 Annual flower export value**

| | 1997 ($ m f.o.b.*) | 1998 ($ m f.o.b.*) | Changes (%) |
|---|---|---|---|
| Roses | 0.62 | 0.53 | - 14.69 |
| Carnations | 0.57 | 0.65 | 14.07 |
| Orchids | 20.53 | 18.93 | - 7.78 |
| Gentians | 0.07 | 0.12 | 78.36 |
| Sandersonia | 3.71 | 3.80 | 2.57 |

* f.o.b. = free on board

**Table 1-2 Gentian exports in New Zealand from 1993-1998***

| Year | Exports ($NZ f.o.b.) | % Change (1993-1994) | % Change (1994-1995) | % Change (1995-1996) | % Change (1996-1997) | % Change (1997-1998) |
|---|---|---|---|---|---|---|
| 1993 | 47,304 | | | | | |
| 1994 | 48,451 | + 2 | | | | |
| 1995 | 68,311 | | + 41 | | | |
| 1996 | 75,089 | | | + 10 | | |
| 1997 | 67,903 | | | | – 10 | |
| 1998 | 120,000 | | | | | + 78 |

* Data collected in this table are from Statistics New Zealand, 1994, 1995, 1996, 1997 and 1998.

## 1.2. Micropropagation and *in vitro* flowering

### 1.2.1. *Plant tissue culture*

The concept of plant tissue culture began in 1838-1839 when Schleiden and Schwann independently stated the basis of cellular theory and implicitly postulated that the cell is capable of autonomy and/or even totipotent. But Schleiden and Schwann had no experimental evidence to show that somatic cells are also capable of the latter (Gautheret, 1983).

Later, when trying to determine experimentally the limit of plant divisibility permitting tissue proliferation, Rechinger (1893) discovered an important approach for plant tissue culture. He used isolated buds, slices of roots, stems, and other materials. The explants were placed on the surface of sand moistened with tap water. He concluded that those pieces thicker than 1.5 mm would develop. But he did not use nutrients or aseptic conditions; his cultures could barely be regarded as tissue cultures. However, Rechinger's experiments led to a concept related to the tissue culture principles; he was a true pioneer in this field (Gautheret, 1983).

The principles of cell culture were clearly formulated nine years later by Haberlandt (1902). Basing on the cell theory, he assumed that there were no limits of divisibility. He chose to work with single cells and used Knop's nutrient solution, sucrose, asparagine and peptone as nutrients. The cells were still alive after 20 to 27 days. They exhibited in the best cases an eleven-fold increase in the original volume but no division.

Following the discovery of auxins by Went (1926), it was demonstrated that this growth substance could enhance cell divisions in plant tissue culture. This has led to the possibility of cultivating plant tissues for unlimited periods, which was eventually announced almost simultaneously by White (1939), Nobecourt (1939) and Gautheret (1939). Later, following the utilization of coconut milk in 1940s, the discovery of

cytokinins in 1961 (Miller, 1961) brought tissue culture into its powerful development. The medium was improved by Murashige and Skoog (1962).

The great breakthrough in clonal propagation can be attributed to Morel (1960) who introduced the technique of meristem culture as a means of vegetative propagation. His method found almost immediate commercial use, and whole new market-oriented plant propagation became available that placed orchids within the economic reach of the average person (Debergh and Maene, 1983). Today the technique developed by Morel is used worldwide and associated with mass production at a competitive price (Debergh and Read, 1991). In modern agriculture and horticulture, it is an important subject to produce plantlets on schedule. The production of plants through tissue culture has the potential use that realizes the mass production of useful crops, especially of ornamental plants.

### 1.2.2. *Micropropagation of ornamentals*

Plant cell and tissue culture techniques have become very powerful tools for propagation of ornamental species. The technology had its beginnings with Haberlandt's speculations regarding cell totipotency at the turn of the 20th century (Hughes, 1980).

Ornamentals are a group of plants where micropropagation has had a tremendous impact, historically, scientifically and economically (Queralt *et al.*, 1991). Ornamental crops include a large variety of different plants, which, in certain countries, make a substantial contribution to the value of the horticultural products. In the Netherlands, for instance, approximately 42% of the total value of agricultural and horticultural products consists of ornamental tuber and bulb crops, pot plants, cut flowers, etc. (Queralt *et al.*, 1991).

Probably orchids were the first horticultural plants to be propagated by tissue culture (Aridity, 1977). It was a major stimulus for application of plant tissue culture techniques to the propagation of ornamental species. This may be attributed not only

to the early work by Morel (1965) on the propagation of orchids in culture, but also to the development and widespread use of a new medium with high concentrations of mineral salts by Murashige and Skoog (1962). Following success with rapid *in vitro* propagation of orchids, there has been an increasing interest in recent years in the application of tissue culture techniques as an alternative means of asexual propagation of economically important fruit, vegetable, flower, forestry and pasture crops (Morel, 1965). Today, plant cell and tissue culture techniques were applied to many other species with varying degrees of success.

Ornamental species from more than 40 different families have been propagated using tissue culture techniques (Queralt *et al.*, 1991), some on a commercial scale. Flowering plant genera in which tissue culture techniques have been successful include Gladiolus, Iris, Chrysanthemums, rose, etc. In United States, many commercial laboratories use tissue culture of shoot tips to propagate a wide range of ornamental plants including orchids, ferns, lilies, etc. (Debergh, 1994).

Actually, tissue culture propagation has been shown to be economically competitive with conventional propagation and has resulted to a large extent in clonal fidelity (Vasil and Vasil, 1980). Two of the most important cut flowers, *Dianthus caryophyllus* and *Chrysanthemum morifolium*, can easily be micropropagated and high propagation rates can be obtained (Queralt *et al.*, 1991).

### 1.2.3. *Micropropagation of Gentians*

Some reports on micropropagation of gentians have been published from the middle of 1980s to middle of 1990s. These include *G. cruciata, G. purpurea, G. punctata* and *G. pannonica* (Wesolowska *et al.*, 1985); *G. lutea* (Lamproye *et al.*, 1987); *G. lutea* (Viola and Franz, 1989); *G. scabra* (Yamada *et al.*, 1991); *G. kurroo* (Sharma *et al.*, 1993).

Micropropagation of more gentian species has been investigated lately (Zhang, 1996, Hosokawa *et al.*, 1996; Morgan *et al.*, 1997; Ivana *et al.*, 1997 and Hosokawa *et al.*, 1998). *In vitro* growth performance of shoots, roots and callus of *G. japonica*, *G. axillariflora*, *G. antarctica*, *G. lutea*, *G. cruciata*, *G. purpurea*, *G. acaulis*, *G. cerina*, *G. corymbifera* and *G.triflora* cultivars cultured in different conditions were described. Woody plant medium (WPM) (Lloyd and McCown, 1980) was first reported to be beneficial to *in vitro* cultures of Gentian species in terms of shoot proliferation and root initiation (Zhang, 1996) as well as callus induction and shoot regeneration (Ivana *et al.*, 1997). B5 (Gamborg *et al.*, 1968) vitamins were also first reported to be advantageous in micropropagation of some gentians species (Zhang, 1996 and Morgan *et al.*, 1997). A concentration of 2% (v/v) sucrose in the medium was found to be the most effective for gentian shoot propagation (Zhang, 1996; Hosokawa *et al.*, 1998). An efficient system for clonal mass propagation in liquid culture was established for the propagation of ornamental gentian (Hosokawa *et al.*, 1998). In this case, the propagation of shoots was maximal after 11 weeks of culture. More than 2000 shoots were obtained in 5 weeks following the initial cultivation of five nodal segments after 6 weeks.

### 1.2.4. *In vitro flowering*

The change from the vegetative state to the reproductive state is one of the most dramatic events in the ontogeny of a plant. Flowering provides for the propagation of individuals and is the vehicle for genetic recombination. Despite the efforts of numerous researchers for over 60 years a satisfactory understanding of the flowering process has not been obtained (Scorza, 1982). However, a few factors involved in the process have been identified.

The process of flowering comprises a series of physiological, biochemical and morphological events including adventitious bud formation, floral bud initiation and flower bud development leading to anthesis (Tanimoto and Harada, 1981a). Once flowering capacity is reached, environmental stimuli such as photoperiod, temperature and nutrition are involved in floral induction. There is a general belief that the

flowering process is chemically controlled, either with a single or multiple factor, a single or multiple inhibitor, or even a combination of these (Carson, 1993). These may include plant growth regulators such as cytokinins, auxins, gibberellins, ethylene, abscisic acid (Raghavan and Jacobs, 1961; Bernier *et al.*, 1977, Franklin *et al.*, 2000), polyamines (Bais *et al.*, 2000), carbohydrates (Roldan *et al.*, 1999; Mitrovic *et al.*, 2000).

### 1.2.4.1 Physical condition of *in vitro* flowering

Flower formation *in vitro* can occur in two ways depending on the state of the parent plant. The first method is when the explants are taken from plants that are completely vegetative. The induction of flowering then occurs in the culture vessel. This has been reported with long day *Cichorium intybus* (Ringe and Nitsch, 1968) and short day *Plumbago indica* (Nitsch and Nitsch, 1967). Alternatively, the explants are taken from plants that are flowering already, such as flower fragments as reported with *Cichorium intybus* (Ringe and Nitsch, 1968), *Nicotiana tabaccum* (Hicks and Sussex, 1970), *Lunaria annua* and *Begonia franconis* (Ring and Nitsch, 1968).

Obviously, floral buds and organ primordia of many species have been cultured *in vitro* since the early 1960. The first attempts to culture floral buds were by LeRue in 1942, with the first reported successful flower culture by Galun and others (1962). Galun showed that floral buds of male, female and bisexual lines of *Cucumis sativus* could be cultured to examine the role of growth substances in the differentiation of sex organs. The first report of the complete development of young excised buds into mature flowers in culture was by Blake (1969) using *Viscaria candida* and *Viscaria cardinalis*. These studies have led to a wealth of information covering mainly the nutritional and hormonal requirements of flower and explant development. Results from many studies have found that young floral buds and inflorescences of a number of species can be grown to maturity (Abernethy, 1996). Research has shown that tendrils, roots, leaf discs, thin sections from stem and inflorescence axis, micro-inflorescence and isolated protoplasts can result in flower formation *in vitro* (Liu and Li, 1989).

*In vitro* flowering has also been obtained from cultures of non-meristematic tissues, by using thin cell layers of *Nicotiana tobaccum* floral shoots (Tran Thanh Van, 1973). Three to six layers of epidermal tissue have been coaxed into various types of organogenesis (buds, shoots and flowers) by growth hormone manipulation, using *Petunia hybrida* (Ramage, 1994). Cotyledons and thin cell layers consisting of epidermal and sub-epidermal cells have also resulted in *in vitro* flowering in tobacco (Mohnen *at al.*, 1990; Mohnen, 1990; Ramage, 1994). The degree of successful regeneration, however, depends on various factors associated with the explant in culture.

### 1.2.4.2 The role of nutrients

Possibly, the most comprehensive investigation of the role of nutrients in *in vitro* flowering is the work of Tanimoto and Harada (1981a,b). These authors found that by manipulating the concentration of salts in the medium and eliminating $NH_4NO_3$ but not necessarily $KNO_3$, flowering could be achieved on nodal segments from induced *Torenia fournieri* Lind plants. Similar works on *Kalanchoe blossfeldiana* Poellniz. (Dickens and van Staden, 1988) supported these findings. This work enlarges on one of the oldest theories of flowering, that nitrogen levels need to be low for flower induction to take place (Kraus and Kraybill, 1918). Tanaka and Asagami (1986) achieved induction of *Lemma paucicostata* Hegelm plant in non-inductive continuous light by transferring from low to high nitrogen levels. Nitrogen was required for the manifestation of the flowering stimulus, but was inhibitory of induction. This was supported by the findings that the suppression of nitrate assimilation by nitrate reductase inhibition also induced flowering (Tanaka and Asagami, 1986).

A greater supply of assimilates increases the likelihood of flower induction within the flower meristem. Techniques which promote flowering such as girdling, high light flux, water or salt stress, addition of gibberellin inhibitors, leaf removal, introduction of cytokinins and dwarfing root stocks can all be explained in terms of carbohydrate to nitrogen levels (Sachs, 1977; Sachs and Hackett, 1977). *In vitro* techniques provide

the ability to manipulate carbon : nitrogen ratios encouraging floral induction, as seen in *Torenia fournieri* (Tanimoto and Harada, 1981c) and *Nicotiana tobaccum* (Nitsch and Nitsch, 1967). Szoke and others (1977) induced *in vitro* flowering on plants regenerated from flower-derived callus cultures on an MS medium supplemented with coconut water, which exhibits a BA-like cytokinin activity. Coconut water has also been used for the induction of *in vitro* flowering from shoot cultures of the bamboo *Dendrocalamus hamiltonii* Munro (Chambers *et al.*, 1991).

### 1.2.4.3 The role of floral promoters and inhibitors

Observations have indicated that the developmental pattern of floral buds and individual organs becomes fixed early in their life cycle (Rastogi and Sawhney, 1987). The stimulus for organ initiation and microsporogenesis and megasporogenesis originates from within the meristem and/or from the other floral organs present at the time of culture. Presumably these influences are mediated through endogenous flower promoters and/or inhibitors. More likely, flowering results from the interaction of plant growth regulators including cytokinins, auxins, gibberellins, ethylene, abscisic acid, or other known or unknown endogenous substances (Raghavan and Jacobs, 1961; Bernier *et al.*, 1977; Wellensiek, 1977). Among these, sugar concentration presumably is a very important one.

#### 1.2.4.3.1 Carbohydrates

Sugars are known to play an important role in culture media for reliable induction and development of flowers (Steinberg, 1950; Margara *et al.*, 1965; Handro, 1977). Glucose, maltose, lactose, and raffinose have been used with success (Margara and Rancillac, 1966; Nitsch and Nitsch, 1967), although sucrose is most commonly used. The optimal sugar concentration for flowering differs among species.

It was found that the endogenous level of free sugars in the apex increases dramatically during induction to flower (Bodson, 1977). It has been argued that high concentrations of carbohydrates within the media induce *in vitro* flowering due to osmotic potential effects rather than the accumulation of sufficient carbohydrates by the plant. However, this has been disproved with work on *Zea mays* cv. Oh43 which requires 10% (w/v) sucrose media for optimal flowering (Pareddy and Greyson, 1989).

The commonly used carbon source is sucrose. A high concentration of sucrose has been found to promote *in vitro* flowering in *Cichorium intybus* (Harada, 1966), *Plumbago indica* (Nitsch and Nitsch, 1967), *Nicotiana tabaccum* (Tran Thanh Van, 1973), *Torenia fournieri* and *Wahlenbergia stricta* (Carson, 1993). Results from these studies have shown that sucrose concentrations of 2% - 4% (w/v) in the media promote the development, but not the initiation, of floral buds (Tanimoto and Harada 1981a). In contrast, cultured tassels of *Zea mays* cv. Oh43 required 10% (w/v) sucrose before normal tassel growth and differentiation occurred (Pareddy and Greyson, 1989).

Sucrose at 2-4% (w/v) stimulated the rate of flowering in *Kalanchoe blossfeldiana*, while concentrations over 4% (w/v) were inhibitory (Dickens and van Staden, 1988). Similar effects of sucrose have also been reported with *Lycopersion esculentun* Mill (Rastogi and Sawhney, 1987), *Bougainvillea* (Steffen *et al.*, 1988) and *Torenia fournieri* (Tanimoto and Harada, 1981b). Rastogi and Sawhney (1987) also investigated the effect of different sucrose concentrations on the development of floral buds. In response to 3% (w/v) sucrose, tomato buds reached maturity. In the absence of sucrose, the buds did not grow at all. In response to 4% (w/v) sucrose, a few flower buds reached maturity with some other buds showing abnormalities in carpels and stamens, while 5% (w/v) sucrose was strongly inhibitory to floral development. Berghoef and Bruinsma (1979) reported that 3% (w/v) sucrose was optimal for the growth of *Begonia* flowers. In bamboo, 2% (w/v) sucrose resulted in 70% of the cultures flowered (Nadgauda *et al.*, 1997). In contrast, 5% (w/v) sucrose was most effective for *in vitro* flowering of Orange Jessamine (Jumin and Nito, 1996). Concentrations of sucrose over 5% depressed *Alstroemaria* flowering (Kristiansen *et al.*, 1999).

#### 1.2.4.3.2 Plant growth regulators (PGRs)

A hormonal substance that functions in the initiation of floral bud formation has been suggested to be biosynthesised in the leaves then transferred to the apical meristems where it acts in the production of floral buds (Hamner and Bonner, 1938, King and Zeevaart, 1973). The name "florigen" was originally coined by Chailakhyan (1936) for a hypothetical substance produced in plants under apporpriate light conditions that induced floral buds in the vegetative apices. Many subsequent attempts to identify this factor chemically by isolating the active principle have been unsuccessful. Tran Thanh Van (1973a) devised an *in vitro* bioassay with explants excised from the cortical layers of tobacco stems. This has the advantage of direct contact of a test sample with tabacco explants in the assay medium and ony 2-3 weeks of culure is needed for formation of floral buds, vegetative buds, roots or callus (Tran Thanh Van, 1973b). From the results of these investigations, it could be concluded that the kind of morphogenetic activity is determined by the auxin/cytokinin ratio. In the study of Tran Thanh Van (1981), kinetin and benzyladenine (BA) were the only cytokinins effective in the initiation of flower buds. This was further investigated by Heylen and Vendrig (1988 and 1991). They reported that a high number of explants with flower buds was found with high cytokinin/2,4-D ratios in thin cell layer cultures of tabacco explants. Actually, numerous experimental studies have shown that plant growth regulators (PGRs), in particular cytokinins and in some cases gibberellins and auxins, play an important role in the initiation, growth and development of various floral organs. PGR requirements vary in different species, different genotypes of the same species, and among different floral organs of an inflorescence.

**(a) Cytokinins**

Cytokinins play an important role in flowering, but it is not known whether this is at the induction, evocation or differentiation stage. Cytokinins were found to inhibit flowering in *Lycopersicon* and *Scrofularia*, but stimulate vegetative growth (Dickens and van Staden, 1990). Joshi and Nadgaauda (1997) reported that in bamboo, BA was found to be the only cytokinin capable of inducing flowering *in vitro*. Flowering was

not observed on media supplemented singly with kinetin, 2iP, zeatin or adenine hemisulphate. Recently, in their research on dynamics of cytokinins in apical shoot meristems of tobacco during floral transition and flower formation. Dewitte *et al.* (1999) concluded that organ formation (e.g. leaves and flowers) is characterised by enhanced cytokinin content. This contrasted with the very low endogenous cytokinin levels found in prefloral transition apices that showed no organogenesis. However, cytokinins do not seem to act as postivie effectors in the prefloral transition phase in tobacco shoot apices.

Research on *in vitro* regulation of organogenesis from tobacco stem internodes led Skoog (1970) to suggest that cytokinins regulate organogenesis by influencing the biosynthesis of other growth factors including thiamine and auxin, and act as modulators in protein biosynthesis. While cytokinins exert a major influence on *in vitro* flowering, the inability of cytokinin to induce flowering from juvenile tissue suggests that an additional factor(s) may be involved (Wardell and Skoog, 1969). Indeed, our understanding of the environmental control flowering in plants has improved in recent years but less is known of the endogenous control. Bernier and coworkers (1977) found that cytokinin applied to apical meristem of *Sinapsis alba* triggered the mitotic cycle that commonly precedes flowering but could not induce subsequent flowering. They hypothesized the existence of a multi-factored flowering stimulus with cytokinin being one component. The concept of a multiple-factor flowering stimulus has gained considerable acceptance (Raghavan and Jacobs, 1961; Evans, 1969; Wellensiek, 1977; Cleland, 1978; Scorza and Janick, 1980). Day *et al.* (1995) observed that in cool conditions, cytokinin concentrations increased, carbohydrate concentrations decreased, flower buds became committed to develop throgh to anthesis and a period of repid bud expansion began. This evidence suggests that cytokinins may influence the rate of flower development by altering the mobilisation of carbohydrates. Some authors indicated that cytokinin might be apart of the signaling system involved in the control of short-day plants flower induction and/or evocation (Galoch *et al.*, 1996). The addition of cytokinins (BAP, kinetin) to isolated apices cultured on suboptimal photoperiods increased flowering. Kinetin can

even induce, in some plants, flowering under quite non-inductive conditions (Galoch *et al.*, 1996).

**(b) Auxins**

*In vitro* studies have implicated auxins as principal floral inhibitors. The effect of auxin on *in vitro* flowering in tobacco has been shown to be a function of the growth regulator concentration. Moderate concentrations of IAA ($3.0\text{-}10^3$ M) decreased the ratio of floral to vegetative buds, whereas higher concentrations inhibited all bud development (Wardell and Skoog, 1969). Pierik (1967a,b) suggested that inhibition of flowering in L. *annua* by IAA was a result of a general inhibitory effect on bud formation. IAA promoted normal petal development on excised buds of *Aquilegia* (Tepfer *et al.*, 1963). A later report by Bilderback (1972) indicated that IAA modified *Aquilegia* floral development, but at high concentrations it was found to be inhibitory.

There are numerous important studies on the relationshisp between IAA and flower formation (Oka *et al.*, 1999). In *Arabidopsis*, for example, the concentration of IAA and the activity of polar auxin transport in the inflorescence axis of the *pin* mutant were much lower than those of the wild type (Okada *et al.*, 1991). Moreover, the application of inhibitors of polar IAA transport to the wild type *Arabidopsis* plant induced the formation of inflorscence axis similar to that of the pin-formed mutant (Okada *et al.*, 1991; Okad and Shimura 1994). These results strongly suggest that normal concentrations of endogenous IAA and/or the activity of polar IAA transport are required for flower formation of *A. thaliana*. These facts were also confirmed by Oka *et al.* (1999) using transgenic *Arabidopsis*.

**(c) Gibberellins**

Gibberellins are regarded as being the most florigenic plant growth regulator, although their promotive effects are mainly confined to cold requiring plants and long day plants with a rosette growth habit. There are however, a substantial number of short day plants that can be induced to flower by gibberellins (Dickens and van Staden, 1990).

Gibberellins applied *in vivo* promote flowering of some species and inhibit flowering of others (Evans, 1971). Gibberellins may induce the change from juvenile to flowering state in some conifers (Pharis *et al.*, 1976) yet cause a reversion to non-flowering and other juvenile characteristics in some angiosperms (Robbins, 1957; Rogler and Hackett, 1975). The effects of gibberellins *in vitro* are that they generally inhibit floral and vegetative bud initiation but have been reported to promote flowering of explants from several species (Chang and Hsing, 1980). Lang (1965) hypothesized that gibberellins affect floral development rather than floral induction. The fact that $GA_3$ inhibits *in vitro* bud formation yet induces "bolting" when applied to flower buds *in vitro* (Wardell and Skoog, 1969; Scorza, 1979) supports this hypothesis. However, most recently, gibberellins have been reported to be positive for *in vitro* flowering in some species, for example, *Trichosanthes dioica* Roxb. (Basu *et al.*, 1999), *Syngonium podophyllum* Schott "White butterfly" (Henny *et al.*, 1999) and *Chenopodium murale* (Mitrovic *et al.*, 2000). Foliar application of $GA_3$ in a dioecious cucurbit *Trichosanthes dioica* could increase the number of female flowers resulting in increased fruit-setting (Basu *et al.*, 1999). Increased $GA_3$ concentration (250 to 2000 mg $l^{-1}$) resulted in increased flower number in *Syngonium podophyllum* (Henny *et al.*, 1999). Similar results were also obtained in some *in vitro* plant tissue cultures lately. $GA_3$ supplemented in the culture medium induced flowering from explants of *Pisum sativum* L (Franklin *et al.*, 2000) and *Chenopodium murale* L (Mitrovic *et al.*, 2000).

**(d) Polyamines**

Polyamines were recognised as plant growth substances only about 10 years ago (Bagni, 1989; Bangi and Torrigiani, 1992). Although the precise physiological functions of polyamines (PAs) have not yet been defined (Havelange *et al.*, 1996), substantial evidence strongly suggestes their interaction with cytokinins in the regulation of the cell division process. Some researchers have suggested that polyamines can mediate the photomorphogenic effect of light or are part of the signal response pathways of plants (Dai and Galston, 1981). The participation of PAs in the control of early steps of the flowering process has been strongly suggested in several

studies involving photoperiodic speceis. In the short-day plants, *Pharbitis nil* cv. Violet, *Xanthium strumarium*, soybean and *Chrysanthemum morifolium*, the titers of various PAs exhibited marked changes in the leaves during photoinduction of flowering (Dai and Wang, 1987; Hamasaki and Galston, 1990; Aribaud and Martin-Tanguy, 1994; Caffaro and Vicentr, 1995). In the long-day plant *Rudbeckia hirta* the titers of free putrescine and spermidine increased both in leaves and apices during floral induction (Harkess *et al.*, 1992). The spermidine availability at the time of flower initiation and organogenesis also might be a factor affecting floral intensity in *Citrus sinensis* L. Osbeck (Ali and Lovatt, 1995). Indeed, polyamines are known to occur in high amounts in the flowers of various plants (Buta and Izac, 1972; Ponchet *et al.*, 1982; Bangi and Torrigiani, 1992; Wada *et al.*, 1994; Scaramagli *et al.*, 1999). Bais *et al.* (2000) found that *in vitro* flowering and the endogenous conjugated pool of polyamines in the shoot cultures of *Cichorium intybus* L. cv. Lucknow local were diminished in response to polyamine inhibitors (DFMA and DFMO) treatments, but could be restored by addition of putrescine and AgNO3 to the shoot multiplication medium. These findings suggest that polyamines may be causally related to flowering, and that conjugated polyamines may constitute potential markers for floral initiation (Daoudi and Bonnet, 1998).

### 1.2.4.4 Photosynthetic photon flux density (PPFD)

Optimum light intensity for plant tissue in culture may differ from the requirements of the plants themselves (Murashige, 1974). Light intensity has been shown to affect the type of growth in culture. Increased light caused an increase in shoot formation in one cultivar of *Begonia* × *hiemalis*, but no increase in dry weight; two other cultivars responded to the increased light with increased biomass but no increase in shoot production (Welander, 1978). Increased PPFD was also reported to have a promoting effect on aerial shoots production of *Alstroemeria* Butterfly-hybrids (Kristiansen *et al.*, 1999).

*In vitro* studies suggest that sugar production through photosynthesis is a key factor. In the dark at low to moderate sucrose levels (0-3%), flowers are not generally produced *in vitro* (Margara and Touraud, 1968; Coleman and Thorpe, 1978). In the dark for as little as two weeks at these sucrose levels (0-3%) can reduce or eliminate subsequent flowering when explants are re-exposed to an inductive light environment (Harada, 1966; Scorza and Janick, 1980). Explants cultured with elevated levels of sucrose (3-5%) have flowered in complete darkness (Henrickson, 1954; Baldev, 1962) and continuous illumination has induced flowering even in the absence of sugar (Margara and Touraud, 1967). Free sugars have been able to substitute for the light requirement for flowering in certain species, such as *Cuscuta reflexa* Roxb. (Baldev, 1959); *Cuscuta reflexa* Roxb. (Baldev, 1962), tobacco (Cusson and TranThanh Van, 1981) and *Kalanchoe blossfeldiana* Poellniz. (Dickens and van Staden, 1988), *Alstroemaria* (Kristiansen *et al.*, 1999).

### 1.2.4.5 pH of the culture medium

The ability of explants to form flowers *in vitro* depends on numerous factors, internal and external, chemical and physical, and virtually all of these factors interact in various complex and unpredictable ways (Tran Thanh Van, 1973; Scorza and Janick, 1980; Lang, 1987; Compton and Vielleux, 1992; Jumin and Ahmad, 1999). Except those important factors we have just mentioned above such as carbohydrates, growth regulators and light intensities, pH of the culture medium is also considered to be important to *in vitro* cultures (Heylen and Vendrig, 1988; Jumin and Ahmad, 1999). Very few reports are available so far on the importance of medium pH to *in vitro* flowering under different light intensities. Although some authors (Perica and Berljak, 1996; Jumin and Ahmad, 1999) reported their investigations of pH effect on *in vitro* flowering, the combined effect of different light intensities with various pH of the culture medium was only reported by Mohnen and others (1990). When light intensity was at 55 $\mu mol\ m^{-1}s^{-1}$ and the pH was at 3.8, tobacco thin cell-layer explants could produce the highest number of flowers *in vitro* (> 60) comparing with vegetative shoots (< 10) and roots (0).

## 1.3. Postharvest life of cut flowers

Many cut flowers deteriorate rapidly after harvest, and short vase life constitutes a major obstacle in the marketing of flowers. Therefore, the postharvest longevity of flowers is of critical importance in determining the value of a cut-flower crop.

### 1.3.1. *Flower senescence*

Senescence in plants is usually viewed as an internally programmed degeneration leading to death. It is a developmental process that occurs in many different tissues and serves different purposes. Traditionally, the senescence mechanisms fall into two major groupings, nutrient deficiencies (e.g., starvation) and genetic programming (i.e., senescence-promoting and senescence-inhibiting genes). Considerable evidence indicates that nutrient deficiencies are not central senescence program components, while increasing evidence supports genetic programming (Noodén *et al.*, 1997).

Research in postharvest physiology of flower crops has resulted in vast improvements in the longevity of flower crops (Woodson, 1991). Discovery that flower senescence is regulated by the phytohormone ethylene has led to the identification and use of chemicals, such as silver thiosulfate, which inhibits ethylene action and delays senescence (Salinger, 1975; Halevy and Kofranek, 1977; Veen, 1979). Development of new treatments that delay senescence or of new cultivars with improved postharvest longevity is currently limited by our basic understanding of the molecular events underlying the regulation of flower senescence.

Loss of cell integrity has been shown to accompany senescence in most plant tissues, including flower organs (Borochov and Woodson, 1989). This is accompanied by increasing production of the phytohormone ethylene, which is known to regulate the process of senescence (Borochov and Woodson, 1989). Sucrose treatment could delay ethylene production, for example, in cut carnation flowers (Dilley and Carpenter,

1975) and sweet pea (Ichimura *et al.*, 1998). The ethylene action inhibitors MCP (1-methylcyclopropene) and DACP (Diazocyclopentadiene) treatments could largely reduce ethylene effects in tomato (Nakatsuka *et al.*, 1997; Tian *et al.*, 1997) and cucumber fruits (Kubo *et al.*, 2000). STS treatment could inhibit ethylene action and improve the post-harvest life of gentian flowers (Zhang and Farr, 1995). Exposure of these flowers to exogenous ethylene hastens the senescence. Zhang and Farr (1995) worked out that the petals of *G. axillariflora* flowers exhibited a characteristic "wilting" behaviour during senescence and in response to exogenous ethylene, i.e. exposure of presenescent gentian flowers to ethylene hastens the onset of petal senescence. Furthermore, treatment of *G. axillariflora* flowers with inhibitors of ethylene action, such as STS or sucrose, prevented typical petal senescence. The "wilting" can be delayed by using STS or sucrose (Zhang, 1996).

Jocelyn Eason and her colleagues at the Crop & Food Research, New Zealand, have isolated 15 genes that are associated with flower senescence (Horticulture News 1997). They found that the gene activities occur at the early stages of senescence, before any deterioration in flower quality can be detected visually. But it is too early to determine their usefulness in extending the vase life of flowers. They are looking for genes regulated by sugar, and are aiming to extend vase life of cut flower via regulation of the activity of these genes. Finding genes that sugars regulate may help to produce flowers with improved post-harvest lives as well, without the aid of pulsing agents. This could be a new way to improve flower longevity at the genetic level.

### 1.3.2. *Carbohydrates in plant tissues*

Carbohydrates in plants are the main energy source for plant growth and cell differentiation, and derived from photosynthesis most. Reserve carbohydrates are stored in the form of starch, a complex polysaccharide. Starch deposition usually occurs in amyloplasts which provide a source of energy for the growth of cellular tissue (Brown, 1988). Osmotic potential is a function of concentration, so assembling 1000 molecules of glucose into one starch macromolecule will reduce the osmotic

potential within the amyloplast by an equivalent factor. This is advantageous as it reduces the strain on various membranes enclosing such macromolecules (Brown, 1988).

Sugars play important role for osmoticum in plant cell. In the intact flower it can be postulated that the osmotic potential is maintained by import of carbohydrate, and presumably the starch acts as a reserve and a buffer against an excessive osmotic potential (Ho and Nichols, 1977). Since the amount of sugars contained in cut flowers is limited, the addition of sugars to vase water is effective in promoting bud opening and extending the vase life of many cut flowers (Mayak*et al.*, 1973; Halevy and mayak, 1979; Paulin and Jamain, 1982; Koyama and Uda, 1994; Kuiper *et al.*, 1995; Ichimura *et al.*, 1998; Ichimura *et al.*, 1999). The continuous presence of sugar in the vase solution may not be required as pulsing cut flower stems with sugar has been shown to be sufficient for the flowers to mature and increase longevity (Salinger, 1985).

### 1.3.3. *Ethylene biosynthesis in higher plants*

Ethylene ($C_2H_4$) is produced by all higher plants. It regulates many aspects of plant growth development and senescence in trace amount. Its effects are often spectacular and are commercially important in agriculture. Ethylene is essential for senescence in many flowers and fruits and exists in the gaseous state under normal physiological conditions. Its biosynthesis involves a multistep enzymatic pathway converting methionine to ethylene. The major route of ethylene synthesis in higher plants involves the following metabolic sequence: methionine → S-adenosylmethionine (AdoMet) or (SAM) → 1-aminocyclo-propane-1-carboxylic acid (ACC) → ethylene (Figure 1-2). In the biosynthesis of ethylene, the conversion of methionine to AdoMet, conversion of AdoMet to ACC and the conversion of ACC to ethylene are catalyzed by AdoMet synthetase, ACC synthase (ACS) and ACC oxidase (ACO), respectively (Mol *et al.*, 1995). Generally, the rate-limiting steps in this pathway are catalyzed by ACC synthase and ACC oxidase, respectively, and most recent literature has dealt with

these two enzymes (Figure 1-2) and the genes encoding them (Van Der Straeten and Van Montagu, 1991).

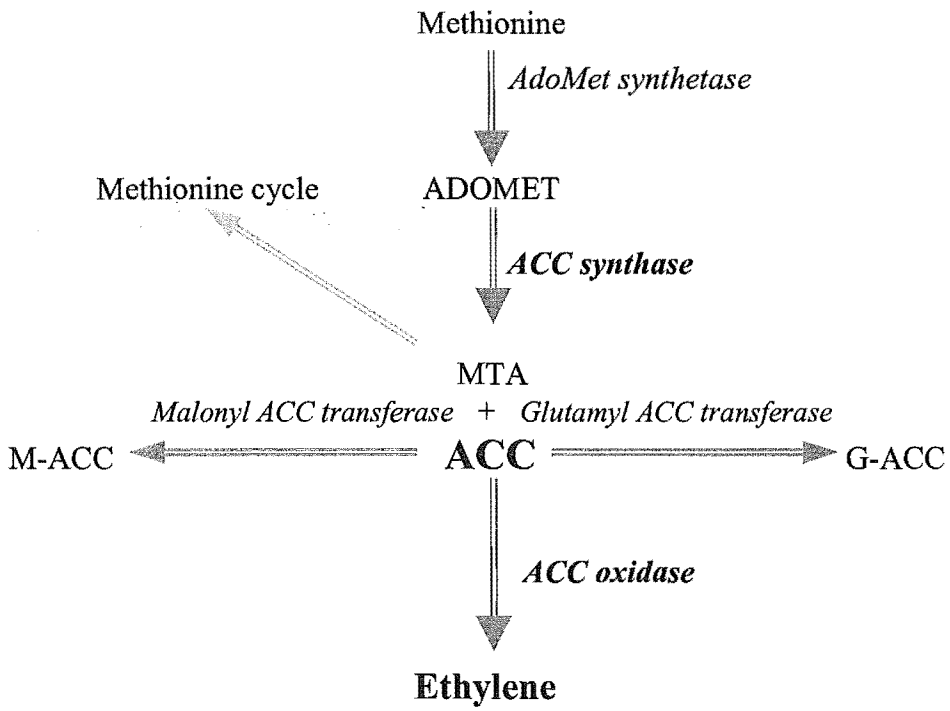


**Figure 1-2** The major pathway of ethylene biosynthesis in higher plants and the enzymes involved. Also shown are routes to methimonie cycle and conjugation reactions that prevent ACC to be directly available for ethylene production. Source: Fluhr and Mattoo (1996).

Following elucidation of the biosynthetic pathway, the enzymes involved have been isolated and characterized (Yang and Hoffman, 1984; Kende, 1993). The different ethylene biosynthesis enzymes are each encoded by a family of genes (Fluhr and Mattoo, 1996). Ethylene markedly induces an increase in the mRNA level of 1-aminocyclopropane-1-carboxylate (ACC) oxidase. To investigate the second messengers that possibly participate in ethylene signalling in mung bean roots, Jung *et al.* (2000) applied various pharmacological reagents known to affect the cytosolic calcium level and phosphoinositide (PI) metabolism the roots. They monitored the induction pattern of ACC oxidase by ethylene as a molecular paradigm for ethylene-dependent responses in this tissue. The results showed that the ACC oxidase enzyme activities and protein levels were in parallel with the abundance of transcript in response to various antagonists and exogenous Ca2+. These results suggested that the PI-derived second messenger and cytosolic calcium are necessarily involved in the ethylene-induced ACC oxidase gene activation in mung bean root tissue (Jung *et al.*, 2000).

The recent progress in research on the molecular aspects of ethylene biosynthesis was based on solid physiological and biochemical work in many laboratories (Kende, 1993). Further important advances in our understanding of the regulatory mechanisms governing ethylene biosynthesis and action are being made. In many instances, the signal that triggers ethylene formation has been identified, e.g. reduced partial pressure of oxygen or auxin (Kende, 1993). In other instances, such as during fruit ripening and flower fading, the signal that causes enhanced ethylene synthesis has yet to be identified (Kadyrzhanova *et al.*, 1997). In addition to the hunt for ethylene signaling components, the means by which the hormone is generated is a subject of intensive study (Johnson and Ecker, 1998). Plants in the vegetative growth phase, flowers, and immature fruits produce barely detectable levels of ethylene until they are subjected to stress or undergo maturation events, after which ethylene production accelerates in a spatially and temporally specific pattern (Kadyrzhanova, 1997).

The simple gas ethylene profoundly influences plants at nearly every stage of growth and development. The use of a genetic approach has been a powerful tool for

investigating the molecular events that underlie these effects. In Arabidopsis, for example, ethylene is sensed by five related recepters (Theologis, 1998). The receptors constitutively suppress a downstram signalling pathway, and are inactivated by ethylene, leading to the activation of genes necessary for the various ehtylene-regualted biological responses (Theologis, 1998). Indeed, several fundamental elements of the pathway have been described: a receptor with homology to bacterial two-component histidine kinases (ETR1), elements of a MAP kinase cascade (CTR1) and a putative transcription factor (EIN3). Taken together, these elements can be assembled into a simple, linear model for ethylene signalling that accounts for most of the well-characterized ethylene mediated responses. (Woeste *et al.*, 1998)

### 1.3.4. *Postharvest techniques*

In the flower industry, quality is determined by several characteristics including stem length, maturity, freedom from defects and vase life. Vase life is one of the most important of these characteristics, as it greatly affects consumer satisfaction. Postharvest treatment is very important for the vase life of cut flowers. Postharvest life of cut flowers depends upon several pre- and postharvest factors, nutritional status of the growth medium, aerial and root temperature, photoperiod and light intensity during the growth period in the greenhouse, conditions during harvesting, grading, packaging, transportation, and composition of 'pulsing' and 'bud opening' solutions (Salunkhe, 1990).

Several major postharvest techniques have been developed and are already in commercial use (Halevy, 1987). These include the use of silver thiosulphate (STS) as an anti-ethylene agent which has been found to extend vase life by totally blocking ethylene action (Salinger, 1975; Halevy and Kofranek, 1977; Veen, 1979); and the application of sugar (sucrose) for the promotion of flower development and longevity (Salinger, 1985). Pulsing cut flower stems with certain amounts of sugar can usually improve flower vase life. It is believed that the principle of pulsing is to fill plant tissues with carbohydrate to ensure that there is sufficient substrate for the flowers to

mature and increase longevity (Salinger, 1985). STS contains silver, which being a heavy metal is a potent environmental pollutant. Therefore, other environmentally sound substances such as sugars are preferable to STS.

Ornamental Gentians are herbaceous perennial plants that are highly valued in the world market as a new cut flower species in recent years. Gentian flowers are ethylene-sensitive as their senescence is hastened by exogenous application of ethylene (Zhang, 1995). Investigations into effective post-harvest treatment of cut gentian flower stems are, therefore, required. Assuming that sucrose pulsing can substitute STS in prolonging the vase life of ethylene-sensitive flowers such as ornamental gentians, it would be tempting to hypothesize that they have similar physiological and biochemical actions in the petals of cut gentian flowers.

It is well known that the anti-ethylene effects of STS include inhibition of ethylene production by the appropriate cut flowers (Veen, 1979). The same anti-ethylene effect has been demonstrated in vase life extension experiments with sucrose (Ichimura *et al.*, 1998). As a vase life promoter, sucrose does delay senescence, this suggests that sucrose plays a key role. In addition, sucrose pulsing has been shown to increase soluble sugar contents of the pulsed petals (Ichimura *et al.*, 1998). However, it remains to be determined if STS pulsing could have the same biochemical effects.

### 1.3.5. *Autocatalytic effect of ethylene biosynthesis*

Ethylene has a positive effect on its own biosynthesis. ACC synthase transcripts can accumulate in tissue treated with ethylene or those tissues that copiously produce ethylene. This is true for tomato (Rottmann *et al.*, 1991; Li *et al.*, 1992; Lincoln *et al.*, 1993), and for carnation (Woodson and Lawton, 1988; Park *et al.*, 1992; Henskens *et al.*, 1994). The accumulation of ACC oxidase transcripts in several cases occurs earlier than that of ACC synthase. Therefore, it would seem that these transcripts are co-ordinately induced by ethylene, causing a very intense autocatalytic ethylene production. ACC oxidase seems to be the first key factor in this phenomenon (Chalutz

*et al.*, 1984): stimulation of ACC oxidase leads to conversion of ACC to ethylene which, in turn, induces ACC synthase transcripts generating more ACC for ethylene production. This is best exemplified by results obtained with floral organs during pollination-induced senescence (Tang *et al.*, 1994). Carnation petals exhibit autocatalytic ethylene production and wilting during senescence. The autocatalytic ethylene production is caused by the expression of ACC synthase and ACC oxidase genes, whereas the wilting of petals is related to the expression of the cysteine proteinase (Cpase) gene (Kosugi *et al.*, 2000). In climacteric fruit, the transition to autocatalytic ethylene production appears to be due to a series of events in which ACC synthase and ACC oxidase genes have been expressed developmentally. Differential expression of ACC synthase and ACC oxidase gene family members is probably involved in such a transition that ultimately controls the onset of fruit ripening (Jiang & Fu, 2000).

## 1.4. Changes in protein and enzyme activities in senescing petals

### 1.4.1. *Changes in protein contents*

In plants, changes in the level of ubiquitinated proteins have been associated with cell death during the development of vascular tissue (Bachmair *et al.*, 1990; Stephenson *et al.*, 1996) as well as during the senescence of leaves and flowers (Courtney *et al.*, 1994; Pinedo*et al.*, 1996; Torre*et al.*, 1999). Loss of protein is a common feature of organ senescence (Hensel *et al.*, 1993; Smart, 1994; Callis, 1995), so it is likely that one component of the program for cell death is the activation of proteinases. In fact, the activity of these enzymes has been correlated with events occurring during death of individual cells and tissues (Tormakangas *et al.*, 1994; Beers and Freeman, 1997) as well as during organ senescence (Ryan and Walker-Simmons, 1981; Callis, 1995). Unlike other plant tissues, it is manifested by an increased reduction in protein content and decline in total activity of the enzyme during petal senescence in some cut flowers such as rose (Faragher and Mayak, 1984; Torre *et al.*, 1999), carnation (Borochov and

Woodson, 1989), petunia (Serek *et al.*, 1995) and daylily (Stephenson & Rubinstein, 1998).

Petal senescence is associated with characteristic biochemical and biophysical events in the tissues, mostly because of alterations in membrane properties that are thought to play a primary role in the senescence process (Borochov and Woodson, 1989). These may include reduced protein content and loosed turgor in the cell. In general, senescing petals exhibit a decline in both phospholipid and protein contents. These changes appear to result from both reduced biosynthesis and increased degradation (Borochov and Woodson, 1989; Itzhaki *et al.*, 1990). Treatment with ethylene decreased protein content by more than 40% and reduced fresh weight in petunia flower petals, and treatment with ethylene action inhibitor 1-methylecyclopropene (1-MCP) was found to be effective in increasing protein content and flower fresh weight (Serek *et al.*, 1995). Calcium ($CaCl_2$) treatments suppressed ethylene production and increased protein level and fresh weight in rose petals (Torre *et al.*, 1999). Similar results were also reported by different authors on alstromeria, begonia, carnation, metthiola, phalaenopsis and phlox (Porat *et al.*, 1995; Serek *et al.*, 1994a,b).

### 1.4.2. *Changes in amylase activities*

Amylase is an enzyme catalyzing the hydrolysis of α-1→ 4 glucosidic linkages of polysaccharides such as starch, glycogen, or their degradation products. It is an endoamylase that attacks the α-1→ 4 linkage at random. The more common α-amylase includes those isolated from *Bacillus subtilis*, *B. Coagulans*, *Aspergillus oryae*, *A. Candidus*, *Pseudomonas saccharophila*, and barley malt. Exoamylases attack the α-1→ 4 linkages only from the reducing ends. Those breaking every alternate bond to produce maltose are known as β-amylase (α-1,4-glucan maltohydrolase). Exoamylases are exclusively of plant or microbial origin (Budavari *et al.*, 1989). In higher plants, in comparison with the extensive range of α-amylases from monocotyledons, only five α-amylases have been reported from dicotyledons, with limited expression data (Wegrzyn *et al.*, 2000).

Amylase activity may be different according to the source materials. This may mainly be affected by addition of EDTA under low pH condition. Some amylases are stable toward EDTA and low pH, and some are not. It is well known that α-amylase appears to require calcium ions for activity (Hsiu *et al.*, 1964; Stein *et al.*, 1964). Some α-amylases, for example, the enzyme from *Aspergillus oryzae* (Fischer *et al.*, 1960), bind calcium very strongly and are resistant to inactivation by EDTA. A component of barley amylases was stable at low pH and EDTA (Paleg, 1960, Frydenberg and Nielsen, 1965; and Jacobsen *et al.*, 1970). Resistance of these enzymes to EDTA and low pH in the absence of added calcium suggests that they do not require calcium ions for activity.

General properties of α-amylases are their insensitivity toward sulfhydryl-oxidizing agents and their strict dependence on $Ca^{2+}$ for enzymatic activity. In the presence of excess $Ca^{2+}$, endosperm α-amylase is stable at 70°C for 20 min (Thoma *et al.*, 1971). It seems that amylases from different sources of plant materials have different properties. Okita and Preiss (1980) reported that the chloroplastic amylase in spinach leaves did not display the above properties. The heating of wheat seeds extracts at 70°C for 15 min decreased α-amylase activity by more than 80% (Battershell and Henry, 1990). The heat stability of the enzyme was probably due to a possible difference in the enzyme structure or the absence of an α-amylase inhibitor which was found in barley.

The specific determination of α-amylase activity in crude plant extracts is difficult because of the presence of β-amylase activity in these tissues that directly interferes with most assay methods. The procedure was developed for the determination of α-amylase activity in barley malt. Determination of the quantities of α-amylase in crude barley malt extracts can be done using selective inactivation of β-amylase by heating the crude extract at 70°C for 20 min in the presence of $Ca^{2+}$ as α-amylase is heat-stable in barley malt (Briggs 1967; Ohlsson, 1926; Olson *et al.* 1939). MacGroegor and others (1971) using ionic exchange chromatography corroborated that treatments of heating at 70°C for 15 min completely inactivated the β-amylase in barley extracts,

without altering the α-amylase properties. On the other hand, determination of the quantities of β-amylase can be done by inactivation of α-amylase by incubation of crude extracts with EDTA, or by carrying out enzyme assay at low pH. These procedures were adopted by several authors (for example, Chrispeels and Varner, 1967; Tarrago and Nicolas, 1976; Monerri and Guardiola, 1986; deMorais and Takaki, 1998). The hydrolytic products can then be examined by chromatographic analyses, e.g. paper chromatography.

### 1.4.3. *Paper chromatography*

A major advantage of paper chromatography, particularly when working with crude tissue extracts, is that they allow an easy detection of product formation in an enzyme assay (Eisenthal and Danson, 1992). This method has been widely used for examination of the products of amylase action in different plant materials such as barley aleurone layers (Jacobsen *et al.*, 1970), spinach leaves (Okita and Preiss, 1980), *Arabidopsis* leaf tissue (Lin *et al.*, 1988) and cotyledons of *Phaseolus vulgaris* (deMorais and Takaki, 1998).

### 1.4.4. *Isozymes*

Isozymes have been recognized for more than 40 years as an important topic for investigations in enzymology and as a major characteristic of the biochemical organization of living systems. The use of the term isozyme to designate multiple molecular forms of enzymes occurring in members of the same species was first introduced into the literature by Markert and Møller (1959). These investigators, on the basis of their own research and after a review of the published research on enzyme heterogeneity, concluded that organisms commonly synthesized enzymes in multiple molecular forms – that is, as 2 or more isozymes. Each isozyme of a given enzyme carried out the same enzymatic reaction but in its own characteristic way to fulfil the specialized metabolic requirements of the cell. Since the first presentation of the concept of isozymes, there has been an explosive growth in the amount of research on

isozymes (Markert, 1974). By now, many thousands of manuscripts have been published in which isozymes have been a major consideration.

Frydenberg and Nielsen (1965) first reported the existence of several isoforms of α-amylase detectable in extracts of barley seeds and many other workers confirmed this observation later (Momotani and Kato, 1966; Jacobsen *et al.*, 1970; Tanaka and Akazawa, 1970). The nature of the isoforms in wheat seeds was characterized (Sargeant and Walker, 1978) using thin layer isoelectric focusing. Much of our knowledge of starch degradation during seed germination comes from studies with cereal (Tarrago and Nicolas, 1976) showing the involvement of a number of amylase isozymes. In contrast, available knowledge of amylase isozymes in flowers is scarce and no paper has been published about amylases in gentian flowers.

## 1.5. Regulation of gene expression and the technical approaches

### 1.5.1. *Polymerase Chain Reaction (PCR)*

The polymerase chain reaction (PCR) is a primer-mediated enzymatic amplification of specifically cloned or genomic deoxyribonucleic acid (DNA) sequences (Innis *et al.*, 1990). It was invented in 1985 by Kary Mullis (Saiki *et al.*, 1985), then working for Cetus Corporation in California, while driving late at night pondering new ways to detect specific bases by DNA sequencing. Indeed, Kary Mullis has given us a fascinating insight into what led to this invention in a *Scientific American* paper (Mullis, 1990). The 1993 Nobel prize for chemistry was awarded to Dr Mullis for having invented PCR.

### 1.5.2. *Scope of PCR application*

PCR is widely used in molecular biology and genetic disease research to identify new genes. Viral targets such as HIV-1 and HCV can be identified and quantitated by PCR. Active gene products can be accurately quantitated using RNA-PCR. In such

fields as anthropology and evolution, sequences of degraded ancient DNAs can be tracked after PCR amplification. With its exquisite sensitivity and high selectivity, PCR has been used for wartime human identification and validated in crime labs for mixed-sample forensic casework. In the realm of plant and animal breeding, PCR techniques are used to screen for traits and to evaluate living four-cell embryos. Environmental and food pathogens can be quickly identified and quantitated at high sensitivity in complex matrices with simple sample preparation techniques (White, 1997). PCR facilitates cloning of DNA sequencing and forms a natural basis for cycle sequencing by the Sanger method (Innis *et al.*, 1988).

### 1.5.3. *RT-PCR*

Reverse transcription PCR (RT-PCR) represents a sensitive and powerful tool for analyzing RNA (Freeman *et al.*, 1999). It is a highly sensitive tool in the study of gene expression at the RNA level and, in particular, in the quantitation of mRNA or viral RNA levels. This technique, also known as message amplification phenotyping (MAPPing), permits the simultaneous analysis of a large number of mRNAs from small numbers of cells (Brenner *et al.*, 1989). RT-PCR can also be used as a first step in preparing a cDNA library by PCR of all of the mRNAs in a sample of cellular RNA. Such methods have been successful and, with continuous improvements being reported, it may be possible to construct a cDNA library from a small number of cells or even a single cell (Newton and Graham, 1997). This would be important in situations where only few cells are available or when the cells of interest cannot be propagated. Definitely, PCR in conjunction with reverse transcription (RT-PCR) can be used to study mRNA almost at the level of a single cell thus allowing investigations that were not previously possible. The result is a revolution in the scale of the nucleic acid manipulations and in the number of samples that can easily be handled.

Reverse transcription is the critical step in RT-PCR experiments. The success of the manipulation relies on the quality of the RNA preparation and on the characteristics of the enzyme used for the reverse transcription. Reverse transcription remains a delicate

manipulation and is, in some cases, difficult to reproduce especially for long retrotranscripts (Edwards *et al.*, 1995).

### 1.5.4. *ACC oxidase genes*

It is clear that flower senescence is a highly regulated developmental event requiring active gene expression and protein synthesis (Lawton *et al.*, 1989). Identification of genes involved in the regulation of senescence could result in the development of genetic engineering strategies to control senescence. The genes encoding ACS and ACO (*acs* and *aco*) have been cloned from many species including carnation, a model system for studying the physiology of flower senescence. Transgenic carnations that contain an antisense *aco* gene have been produced using a carnation *aco* cDNA clone (Mol *et al.*, 1995). When Scania and White Sim cultivars of carnations were transformed with an antisense *aco* gene, their flowers produced low levels of ethylene and exhibited a marked delay in petal senescence (Mol *et al.*, 1995). Ethylene production is tightly regulated, primarily through expression of the ACC synthase family of enzymes. The recent identification of the ethylene overproducer *eto2* gene illustrates the importance of these genes in controlling ethylene production (Vogel *et al.*, 1998).

Ethylene elicits diverse physiological responses and induces specific changes in gene expression during a broad spectrum in a plant life cycle (Chae *et al.*, 2000). A number of developmental pathways in plants result from the activation of generically defined programs that are under the control of ethylene. These include seed germination, leaf abscission, fruit ripening, leaf and flower senescence, as well as responses to environmental stress such as wounding, anaerobiosis, drought, flooding or pathogen attack (Yang and Hoffman, 1984; Abeles *et al.*, 1992). In recent years, much attention has been devoted to the isolation of genes involved in the biosynthetic pathway of ethylene (Kende, 1993; Yang and Dong, 1993; Zarembinski and Theologis, 1994) and the mechanism of its action (Kieber and Ecker, 1993; Ecker, 1995). The last two steps of the biosynthetic pathway are under the control of ACC synthase and ACC oxidase (Figure 1-2). ACC synthase catalyses the conversion of S-adenosylmethionine to 1-

amino-cyclopropane-1-carboxylic acid (ACC), which is then converted to ethylene by ACC oxidase. Since ACC synthase is generally regarded as the rate-limiting step in ethylene (Yang and Hoffman, 1984; Zarembinski and Theologis, 1994), work has focused on the regulation of ACC synthase gene expression. It is known that ACC synthase is encoded by a multigene family, each gene being differentially regulated during plant development (Theologis, 1992) and by the various stimuli known to induce ethylene production (Nakajima *et al.*, 1990; Olson *et al.*, 1991; Spanu *et al.*, 1993; Kende and Zeevart, 1997).

As is the case with ACC synthase, ACC oxidase is found to be encoded by a gene family (Kende, 1993; Kend and Zeevart, 1997). Recent molecular studies have shown that ACC oxidase gene family is differentially expressed in different organs, and at distinct developmental and physical conditions (Kende and Zeevart, 1997). For example, three ACC oxidase genes were identified from both tomato and melon plants (Barry *et al.*, 1996; Lasserre *et al.*, 1996). The expression of each member of gene family is spatially and developmentally regulated in a gene-specific manner in wounded and ethylene-treated leaves, during the senescence of flowers and leaves, in the ripening process of fruits, and in response to pathogen attack (Barry *et al.*, 1996; Lasserre *et al.*, 1997; Blume and Grierson, 1997). In tomato, the ACC oxidase transcripts are spatially regulated throughout flower development: *ACO1* gene was expressed predominantly in senescing petals, stigma and style, while *ACO2* expression is mainly restricted to tissues associated with the anther and the *ACO3* transcripts accumulate in all of the floral organs examined apart from the sepals (Barry *et al.*, 1996). Liu *et al.* (1997) have shown that three ACC oxidase homologues, *ACCO1*, *ACCO2* and *ACCO3*, are differentially regulated in sunflower organs, and are induced by wounding and silver ion treatment in seedlings. More recently, Hunter and others (1999) have examined the expression of three ACC oxidase genes during leaf ontogeny in white clover. The results have revealed that *TRACO1* is expressed specifically in the apex, and *TRACO2* in the apex and in developing and mature green leaves, while the third gene, *TRACO3*, is active in senescent leaf tissue. These results provide evidence that ACC oxidase genes are not constitutive, but the specific expression of each member of gene family is critical for the regulation of ethylene

biosynthesis in vegetative tissues, as in ripening fruits and flowers. Kosugi and others (2000) investigated the expression of these genes in petals of a transgenic carnation harboring a sense ACC oxidase transgene and in petals of carnation flowers treated with DPSS [1,1-dimethyl-4-(phenylsulfonyl)semicarbazide]. In petals of the transgenic carnation flowers, treatment with exogenous ethylene caused accumulation of the transcript for cysteine proteinase (Cpase) and wilting, whereas it caused no or little accumulation of the transcripts for ACC oxidase and ACC synthase and negligible ethylene production. In the petals treated with DPSS, accumulation of the transcripts for ACC synthase and ACC oxidase was found, but there was no significant change in the level of the transcript for CPase. These results suggest that the expression of ACC synthase and ACC oxidase genes leading to ethylene production is differentially regulated from the expression of Cpase, which is associated with wilting in carnation petals.

Studies of the regulation of expression based on transcript analysis show an accumulation of mRNA during ripening, after wounding of leaves (Holdsworth *et al.*, 1988; Balague *et al.*, 1993), ethylene treatment of flowers (Tang *et al.*, 1994), and elicitor treatment (Felix *et al.*, 1991). However, very little is known about differential expression of ACC oxidase genes. So far, only two multigenic families encoding ACC oxidase have been isolated and characterized. Three genes were found in tomato (Holdsworth *et al.*, 1988) and four, including a pseudogene, in petunia (Tang *et al.*, 1993). In tomato, all three genes were shown to be transcriptionally expressed (Bouzayen *et al.*, 1993). Differential expression has been reported in petunia flowers, *PH-ACO1* being expressed specifically in senescing corollas, *PH-ACO3* and *PH-ACO4* in developing pistil tissue (Tang *et al.*, 1994). In broccoli, two ACC oxidase genes isolated from senescing florets were found to be differentially expressed (Pogson *et al.*, 1995). In other recent work, enhancement of ethylene production by indole acetic acid (IAA) was shown to occur via induction of ACC oxidase gene transcription by ethylene itself. This raises the possibility that, in this instance, ACC oxidase is a rate-limiting enzyme in ethylene biosynthesis (Peck and Kende, 1995). Taken together, these results show that expression of ACC oxidase genes is not constitutive

and that control of their transcription may play a role in regulating the rate of ethylene production.

The enzyme that converts ACC to ethylene is encoded by multigene families in several plant species. Whereas control of ethylene production is largely attributed to ACC synthesis (ACS), the differential expression patterns of ACC oxidase (ACO) genes suggest that oxidases contribute to regulation of ethylene production as well. Periods of ACO induction correlate with ethylene-regulated events in several instances, including senescence, fruit ripening, and wounding (Johnson and Ecker, 1998). In addition, ACO and ACS together contribute to the positive feedback loop wherein ethylene treatment leads to increased ethylene production (Kende and Zeevart, 1997).

## 1.6. The objective of this study

Gentian is a new cut flower crop grown in New Zealand. The production is continually increasing year by year. Research on the crop includes various aspects but particularly important one is to improve flower longevity, which is with specific commercial importance to New Zealand flower industry. The overall aim of this study is to increase our understanding of the critical events leading to the gentian flower senescence. To this end, this study would focus on:

1. To establish a reliable *in vitro* flowering system for further studies.
2. To determine the postharvest life of cut gentian flowers with respect to white bud development, blue bud opening and flower petal wilting in response to pulsing treatments with carbohydrates or STS.
3. To investigate physiological changes in gentian flowers such as ethylene production, soluble sugar and starch content changes in the petals during flower senescence in response to pulsing treatments with sucrose or STS.
4. To identify potential biochemical markers, eg. protein-based-markers that may be associated with the physiological changes in petal senescence of *G. triflora*.
5. To initiate a molecular study approach towards the isolation of ACC oxidase (ACO) gene specifically expressed during flower senescence in *G. triflora*.

## 1.7. Justification of the contents of thesis

Chapter one is the introduction where the relevant published literature was reviewed and the objectives were given for present study. Chapters two to four are materials and methods, results and discussion, respectively, where the fine points of the experimental approaches and results were presented and were discussed in details. The last chapter, Chapter Five, refined the discussion, and the conclusions were given for the overall study in this chapter. The relationship of each objective and the flower sources are indicated in Figure 1-3.

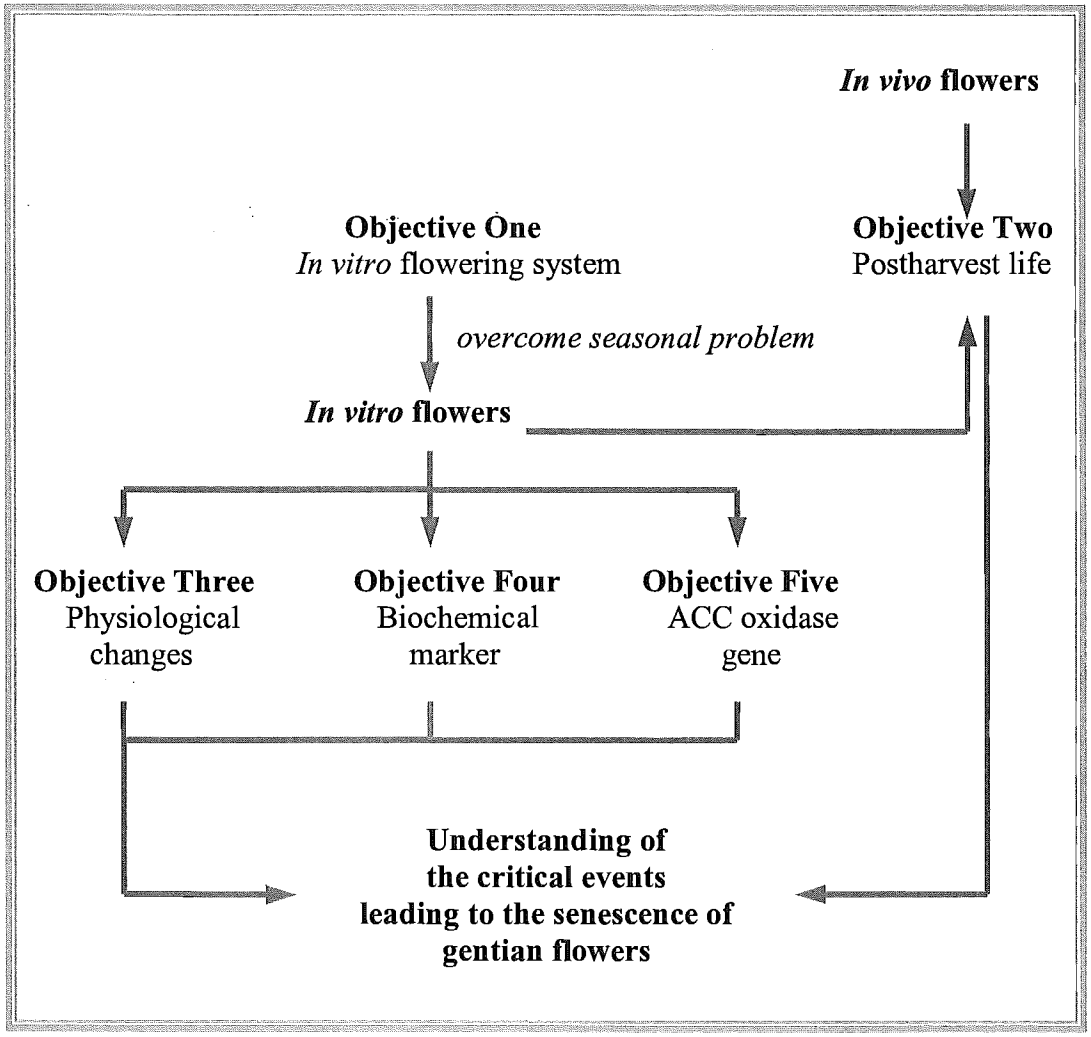


**Figure 1-3 Diagrammatic representation of the relationship of each objective and plant material adopted in the study**

# Chapter 2. Material and Methods

## 2.1. Reagents

The key chemicals in this study were obtained from following companies: **BDH** chemicals Ltd, Pool, England; **BIO-RAD**, Hercules, CA, USA; **Roche Molecular Biochemicals, BOEHRINGER MANNHWEIM**, GmbH, Germany; **LIFE TECHNOLOGIES™**, Gaithersburg, MD, USA; **SIGMA®**, St. Louis, MO, USA and **GIBCOBRL Life Technologies LTD**, Auckland, New Zealand.

## 2.2. *In vitro* flower system

### 2.2.1. *Plant material and in vitro shoot multiplication*

Plant material of *Gentiana triflora* Pall. var. *axillariflora* Akita Blue was obtained from a local gentian farm in Canterbury, New Zealand. The young shoots of *G. triflora* were selected and washed with tap water for one hour. After dipping in 70% (v/v) ethanol for 30 seconds, the shoots were surface disinfected in 20% (v/v) household bleach (Dynawhite, Wilson Chemicals Ltd. New Zealand) containing 4.8 ± 0.2% sodium hypochlorite (NaOCl) for 30 min, rinsed 5 times in sterile distilled water, and cut into single nodal sections each 15–20 mm long which were cultured in 250ml polycarbonate tissue culture vessels (Biolab Scientific Ltd. Auckland, New Zealand) each containing 50 ml medium. A modified method was used as described by Zhang (1996) for *in vitro* shoot multiplication in a culture room at 22°C, with continuous lighting at 30μmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux density (PPFD) provided by

white fluorescent tubes. The lower end of the nodal segment was inserted into double-strength WPM medium (Lloyd and McCown, 1980) containing 2% sucrose (w/v) and 0.75% agar (w/v) (Bacteriological agar, Germantown company, New Zealand). The pH of the medium was adjusted to 5.7 with 0.1M NaOH before autoclaving at 121°C under 137kPa for 20 min. Benzyladenine (BA) dissolved in DMSO was added to the autoclaved medium at the concentration of 0.5 mg $l^{-1}$. After 10 weeks, the *in vitro* shoots were long enough for the following experiments.

### 2.2.2. *Flowering gradient*

The *in vitro* shoots were subdivided starting from the apex into five sequential nodal segments each 15-20 mm long (Figure 2-1). A single explant was planted upright in a 250 ml polycarbonate tissue culture vessel containing the same medium and culture condition for *in vitro* shoot multiplication. The number of nodal explants forming shoots and flowers was determined after 5 and 12 weeks, respectively. In each treatment, there were 10 replicate nodal explants and all experiments had been conducted three times.

### 2.2.3. *Effect of $GA_3$*

The BA in the medium for *in vitro* shoot multiplication was replaced by different $GA_3$ concentrations ranging from 0 to 0.2 mg $l^{-1}$. In this and the following experiments, 10-week-old *in vitro* gentian shoots were subdivided starting from the apex into 3 sequential nodal segments each 15-20 mm long. A single explant, chosen randomly from the isolated nodal segments as described, was planted upright in the appropriate medium in a 250 ml polycarbonate tissue culture container. The cultures were maintained in a culture room at 22°C, with a continuous lighting at 60 μmol $m^{-2}$ $s^{-1}$ of PPFD.


**Figure 2-1 Nodal segments excised from an *in vitro* shoot of *G. triflora***

*In vitro* shoot of *G. triflora* was cut into nodal explants from 1 to 5 (apex to base). These nodal segments were used for *in vitro* shoot and flower investigations as described in the text.

### 2.2.4. *Effect of pre-incubation in the dark*

The cultures of young nodal explants were placed in a dark room at room temperature for 5 weeks before they were transferred to a culture room under a flowering inductive light condition, i.e. continuous lighting of PPFD at 60 μmol $m^{-2}$ $s^{-1}$, provided by white fluorescent tubes for 7 weeks. Alternatively, the cultures were kept in the light for 12 weeks. In this experiment, the double-strength WPM basal medium was supplemented with 0.5 mg $l^{-1}$ BA and sucrose concentrations ranging from 1-5% (w/v).

### 2.2.5. *Interaction between sucrose and light intensity*

The effect of different sucrose concentrations ranging from 1-5% (w/v) plus sugar-free control, on *in vitro* shoot and flower formation of *G. triflora* was tested with continuous lighting of different PPFD, at 30, 60 and 120 μmol $m^{-2}$ $s^{-1}$, provided by white fluorescent tubes. In this experiment, the base medium was the double-strength WPM basal medium supplemented with 0.5 mg $l^{-1}$ BA.

### 2.2.6. *Interaction between BA and light intensity*

BA at concentrations from 0 to 1.0 mg $l^{-1}$ were tested for *in vitro* shoot and flower formation of *G. triflora* under continuous lighting of PPFD at 60 μmol $m^{-2}$ $s^{-1}$ provided by white fluorescent tubes. The appropriate BA concentration was added to the double-strength WPM basal medium supplemented with 2 % (w/v) sucrose.

### 2.2.7. *Interaction between pH and light intensity*

The double-strength WPM basal medium consisting of 2 % (w/v) sucrose and 0.5 mg $l^{-1}$ BA was adjusted to different pH values: 3.7, 4.7, 5.7, 6.7 and 7.7 before autoclaving. The effect of these media on *in vitro* shoot and flower formation was recorded after 5 and 12 weeks of culture, respectively.

## 2.2.8. *The comparison of in vitro and in vivo flowers*

The *in vitro* flowers were compared with those collected from the gentian flower farm (herein referred to as "*in vivo*" flowers). The *in vitro* and *in vivo* flowers were compared in terms of flower size, shape and colour, as well as anther number, pollen size and viability.

### 2.2.8.1 SEM examination of pollen grains and seed-like structure

Scanning electron microscope (SEM) observations were conducted for the pollen and the seed-like structure of *G. triflora*. Specimens from both *in vitro* and *in vivo* source were mounted on stubs for coating. A diode splutter (Polaron E5000) at 50 mA and 1.2 kV was used to coat the samples with gold. Over 5 minutes the splutter deposited 500 nm of gold. Electron scanner (LFTCA S440, Cambridge Instruments Ltd, Cambridge, England) operation was conducted using secondary electrons at 50 to 100 pA probe current and 5 kV EHT.

### 2.2.8.2 Viability test of the pollen grains

The anthers were removed from both *in vitro* and *in vivo* flowers of *G. triflora* to obtain pollen for viability test using the Alexander's procedure (Alexander, 1969). The stain was prepared by adding the various constituents in the order as follows: 10 ml of 95% alcohol, 10 mg of Malachite green (Aniline) (1 ml of 1% solution in 95% alcohol), 50 ml of distilled water, 25 ml of glycerol, 5 gm of phenol, 5 gm of chloral hydrate, 50 mg of fuchsin acid (5 ml of 1% solution in water), 5 mg of orange G (0.5 ml of 1% solution in water) and 1–4 ml of glacial acetic acid. Pollen specimens from both *in vitro* and *in vivo* flowers were mounted directly in a drop of the stain separately, covered with a cover slip, warmed over a small flame, and examined under a microscope (Olympus CHK). Photos were taken when required under a photomicrographic system (Olympus Biological Microscope BH2 with Olympus Automatic Photomicrographic System PM-10ADS).

#### 2.2.8.3 Germination test of the pollen grains

WPM medium with 10% (w/v) sucrose and 1% agar was used for pollen germination test. Pollen grains from either *in vitro* or *in vivo* flowers were cultured on the medium in Petri dishes (50 mm in diameter), with 3 replicates, at 23°C in the dark and observed every 30 min under a stereomicroscope.

#### 2.2.8.4 Pollination test

Hand pollination for both *in vitro* and *in vivo* flowers were conducted to examine the capacity of both types of plants for seed production. For pollination of *in vitro* plants, pollen grains were transferred from anther to stigma with a disinfected small brush in a lamina flow cabinet, while several gentian plants grown in the local gentian farm were similarly hand pollinated.

### 2.2.9. *Statistical analysis*

The test of homogeneity, Chi-square ($\chi^2$), was used for comparison of binomial data expressed as decimal fractions or percentages using Analytical Software Statistix for Windows 3.6. A fixed model one-way analysis of variance (ANOVA) was used for analysis of the effect of the treatments on the production of flowers and shoots *in vitro*. Data were presented using Scientific Graphing Software "SigmaPlot®", Version 2.0 (Jandel Corporation).

## 2.3. Study on post-harvest life of cut gentian flowers

### 2.3.1. *Vase life assessment*

#### 2.3.1.1 Plant materials

Gentian flowers were freshly harvested from a local grower in Canterbury, New Zealand. The stems used were 60 cm long with 8–10 leaf pairs, 10–15 open flowers and 12–15 buds (blue and white).

### 2.3.1.2 Sugar pulsing treatments

(1) The effects of pulsing with different sucrose concentrations ranging from 1 to 10% (w/v) were tested. (2) The effects of pulsing with fructose, glucose or sorbitol singly were tested at one concentration, 87.6 mM which is osmotically equivalent to 3% sucrose (w/v). The cut end of the flower stems were placed in 1000-ml Erlenmeyer flasks each containing 200 ml of a test solution plus 200 mg $l^{-1}$ 8-hydroxyquinoline sulphate (HQS) (Sigma St Louis USA) at room temperature for 24 hours. HQS dissolved in water (200 mg $l^{-1}$) was used as control. HQS was recommended as a germicide in a vase solution by Zagory and Reid (1986).

### 2.3.1.3 STS pulsing treatments

The effects of STS at different concentrations ranging from 0.2 to 5.0 mM were tested. A stock solution of 100 ml STS was prepared as follows: 1.7g silver nitrate ($AgNO_3$) (BDH Poole England) and 20 g sodium thiosulphate ($Na_2S_2O_3 \cdot 5H_2O$) (Sigma St Louis USA) were dissolved in 50 ml de-ionized water each separately, before the silver nitrate solution was added with continuous stirring to the sodium thiosulphate solution. The resulting 100 ml of stock solution contained approximately 0.1M $AgNO_3$ and 0.8M $Na_2S_2O_3 \cdot 5H_2O$ with a ratio of silver (Ag) to thiosulphate ($S_2O_3$) of 1:8, which is generally regarded as optimum for treating flowers (Denise Farr, Lincoln University, Canterbury, New Zealand, personal communication). STS pulsing solutions were made by, for example, taking 2 ml of stock solution and diluted to 1000 ml to give a solution with 0.2 mM silver. The flower stems were pulsed with these solutions in 1000 ml Erlenmeyer flasks each containing 200 ml of a test solution with 200 mg $l^{-1}$ HQS at room temperature for 24 hours.

### 2.3.1.4 Vase life appraisal

Immediately after pulsing treatment, the stems were transferred to flasks containing de-ionized water with 200 mg $l^{-1}$ of HQS for vase life assessment in a growth room at 22°C, 70% relative humidity, and 16 hours of illumination daily at a photosynthetic photon flux density (PPFD) of 60 μmol $m^{-2}$ $s^{-1}$ using cool white fluorescent tubes.

Each stem was inspected visually to determine the number of white buds turning blue, blue buds opening and open flowers becoming wilted every day. The vase life of open flowers is considered over when the petals began to lose their turgor.

## 2.3.2. *Changes in carbohydrate contents*

### 2.3.2.1 Plant materials

*In vitro* flowers of *G. triflora* were collected freshly at the anthesis in Plant Biotechnology Laboratory, Department of Plant and Microbial Sciences, University of Canterbury, New Zealand. The flowers were treated with sucrose, fructose, glucose and sorbitol each at 87.6 mM or STS at 0.5 mM for 24 hours at room temperature.

### 2.3.2.2 Extraction of petal

The petals of *in vitro* flowers from the different treatments were placed into a pre-chilled mortar and grounded to a fine powder with liquid nitrogen. In a total volume of 5.0 ml of 80% (v/v) ethanol, 50 mg of fine powder was extracted and then centrifuged at 11000 rpm for 10 minutes at 2°C [Eppendorf refrigerated centrifuge (5403)]. Two fractions were obtained for the soluble sugar and starch assays.

### 2.3.2.3 Soluble sugar assay

The soluble sugar content of the supernatant after centrifugation was determined (Dubios *et al.*, 1956). To 0.5 ml of the test solution, 0.5 ml of 5% (w/v) phenol and 1.0 ml of concentrated $H_2SO_4$ were added to the solution and mixed thoroughly. The absorbance of each sample was read at 490 nm (Zenspec II spectrophotometer) using glucose as a standard.

### 2.3.2.4 Starch assay

The starch content of the pellet after centrifugation was estimated using a method described by Bewley *et al.* (1993). Starch was extracted from the resulting pellet with

6.0 ml of 30% (v/v) perchloric acid. The slurry was left at room temperature for 24 hours. The amount of amylose was estimated using an iodine reagent, which was freshly prepared by mixing 0.1 ml iodine stock with 9.9 ml of 0.05 M HCl. To 0.5 ml of the starch solution, 0.5 ml of iodine reagent and 1.0 ml of 30% (v/v) perchloric acid were added and the solution was vortexed. The absorbance of each reaction mixture was measured at 620 nm (Zenspec II spectrophotometer), using soluble potato starch as a standard.

### 2.3.3. *Ethylene production*

*In vitro* flowers were pulsed with water, 3% sucrose or 0.5 mM STS solutions for 24 hours before they were transferred to new jars containing only water for ethylene measurement over a period of 14 days. Individual petals isolated from intact flowers after the respective treatments were enclosed in 250 ml jars with a rubber septum. Following a 2-hour incubation period at room temperature, 2 ml gas samples were withdrawn into a syringe and ethylene concentration was analyzed using a gas chromatograph (HP 6890) equipped with an activated alumina column and a flame ionization detector.

### 2.3.4. *Autocatalytic ethylene production*

The modified method of Woodson and Lawton (1988) for autocatalytic ethylene production measurement was used. *In vitro* flowers were removed from the polycarbonate tissue culture containers and then equilibrated in air for 15 min with the cut end inserted into a small vial containing $dH_2O$. The flowers were treated with water, 3% sucrose or 0.5 mM STS for 24 hours prior to the initiation of ethylene treatments. The flowers were placed in 1 L jars, and ethylene was injected into the jars through a rubber septum in the lid using a hypodermic needle to a final concentration of 20 $\mu l\ l^{-1}$ (20 $\mu$l of 1000 mg $l^{-1}$ standard ethylene to each 1 L jar). Saturated KOH was placed in the jar with a paper wick to absorb evolved $CO_2$. Control flowers were enclosed in jars without added ethylene. The flowers were exposed to exogenous ethylene for 24 hours. After this, petals were detached from the flowers and equilibrated with air for 15 min before 0.5 g of petals were placed in a 5 ml serum vial.

Preliminary tests showed that a 30-min-accumulation-time in the vials was the optimal time for ethylene collection. After 30 min 1 ml gas sample was withdrawn using a syringe and ethylene concentration was analyzed using a gas chromatograph (HP 6890) equipped with an activated alumina column and a flame ionization detector. The amounts of ethylene produced were calculated according to the fresh weight of petal (g), incubation time (h) and the concentration (nl) converted from peak readings (pA) from GC against ethylene standard curve:

$$y = 0.2119x + 0.0286$$

$$C_{(nl/gfw/h)} = (pA - 0.0286 / 0.2119) / g / h$$

### 2.3.5. *Radioactive studies*

[U-$^{14}$C]Sucrose at 7.40 MBq/ml (200 μCi/ml) was purchased from Amersham Pharmacia Biotech UK Limited. Method used here followed the instruction provided by Amersham Pharmacia Biotechnology UK Ltd. Gentian flowers were freshly collected at their anthesis stage. Individual flowers were randomly chosen and put into a 10ml-vial for pulsing for 24 h with 5 ml of 87.6 mM sucrose solution plus 78 KBq $^{14}$C-sucrose. The experiment was conducted twice. The $^{14}$C labelled petals were weighed and ground into a fine powder for soluble sugar and starch determination as described earlier here. Four replicate aliquots of 0.3 ml each of the ethanol-soluble fractions or 0.1 ml each of the perchloric acid extracts of the ethanol-insoluble fractions were placed in scintillation vials. To each vial, 5 ml liquid scintillation cocktail (Amersham Pharmacia Biotech UK Ltd.) were added, and then all the vials were left in the dark for 2 hours at room temperature. A liquid scintillation counter (Wallac 1410) was programmed to detect $^{14}$C radioactivity.

### 2.3.6. *Statistical analysis*

Using Analytical Software Statistix 3.7 for Windows, a fixed model one-way analysis of variance (ANOVA) was used for analysis of the data from different treatments in an experiment. The results were then compared using Tukey's multiple comparison test,

except the $^{14}C$ experiment in which the significant differences between 2 treatments were tested using Student's t-test tests. Treatments are presented using Microsoft® Excel 97 (Microsoft Corporation) and Scientific Graphing Software "SigmaPlot®", Version 2.0 (Jandel Corporation).

## 2.4. Biochemical changes in petal senescence

### 2.4.1. *Plant material*

Fresh *in vitro* flowers at different stages, e.g. blue buds, opened flowers and wilted flowers, were collected in the Plant Biotechnology Laboratory, Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand.

### 2.4.2. *Pulsing with sucrose and STS*

Flowers were collected freshly and pulsed with 3% (w/v) sucrose or 0.5 mM STS solutions for 24 hours at room temperature, respectively. As a control, one group of flowers was pulsed with de-ionized water for 24 hours at room temperature.

### 2.4.3. *Preparation of crude extracts (CE)*

The gentian flowers treated with 3% (w/v) sucrose, 0.5 mM STS or $dH_2O$ (control) and the petals, sepals and reproductive parts were weighed and homogenized in liquid $N_2$. One ml of 0.1 M sodium phosphate buffer (pH 7.0) (Appendix A1) was added to each sample, in an Eppendorf tube, and mixed well. After incubating for 30 min on ice the samples in Eppendorf tubes were centrifuged at 11000 rpm for 5 min at 4°C. The supernatants were stored at – 20°C.

### 2.4.4. *Total amylase assay*

The optimum concentration of crude enzyme (CE) used for total amylase assay were first determined from preliminary trials. For the total amylase assay, tube I was set as control containing 600 µl of 0.2 M acetate buffer (pH 5.2) (Appendix A2) and 50 µl

CE. Tube II contained 350 µl acetate buffer and 50 µl CE. In tube III, 350 µl acetate buffer, 50 µl CE and 200µl of 0.2% starch were added. After vortexing, the mixtures were incubated at 37°C for 15, 30, 45 and 60 min in a water bath. After this, all the tubes were placed on ice. To the tube II, 200 µl of 0.2 % starch were added, 3 ml of iodine reagent to each tube to stop the enzyme reaction before absorbance was read at 620 nm using a spectrophotometer (Zenspec II). All the tubes were in triplicates.

To investigate amylase activity at optimum pH, sodium phosphate buffer at pH from 4 – 8 (Appendix A1) were used in the assay reaction mixtures.

### 2.4.5. *Native Page of amylase*

Before running native page gels, separating gel, stacking gel and electrophoresis buffer were made following the recipes shown in Appendix B1, B2 and B3, respectively. CE (20 µl) was mixed with 10 µl of 60% sucrose and 5 µl of 0.003% bromophenol blue, and loaded onto the wells. The gels were run at constant 120 V in a cold room (4°C) for about one hour. The gels were removed from the mini-gel apparatus and then incubated in 0.5% soluble potato starch solution at 37°C for 30 min before they were stained with the iodine reagents (Appendixes A3 and A4) for 10 min in darkness.

### 2.4.6. *Type of amylase assay*

Two sets of boiling tubes were placed on ice with 50 µl CE prepared from the petals, sepals and reproductive parts of gentian flowers. Aliquots of 350 µl acetate buffer each were added to tube I and tube II but 600 µl to a control tube. After this, 50 µl of the crude extracts was added to each of the tubes I and II. The mixtures were vortexed and incubated at 70°C in a water both for 5 min. The tubes were then placed in ice and an aliquot of 200 µl of 0.2% starch was added to tube II. After incubation at 37°C in a water bath for 15 min, the tubes were placed in ice and an aliquot of 200 µl of 0.2% starch was added to the tube I. Finally, 3 ml iodine reagent (Appendix A3) were freshly prepared from iodine stock (Appendix A4) and added to each tube. The absorbance was read at 620 nm using a spectrophotometer (Zenspec II).

## 2.4.7. *Determination of hydrolysis products by paper chromatography (PC)*

### 2.4.7.1 Solvent system (Scott, 1969)

The solvent system for paper chromatography was a mixture of ethyl acetate / pyridine / water (12:4:3, v/v).

### 2.4.7.2 Sugar detection reagents (Scott, 1969)

The spray reagent for sugar detection on paper chromatograms was a mixture of diphenylamine / aniline / phosphoric acid. This was prepared as in the following: (1) 4 grams of diphenylamine were dissolved in 80 ml acetone before being made up to 100 ml with more acetone; (2) 4 ml aniline were added to 96 ml acetone and mixed well; (3) 20 ml of 85% ortho-phosphoric acid. These solutions were mixed just prior to use.

### 2.4.7.3 Starch hydrolysis by crude extracts from the flower parts

#### 2.4.7.3.1 Total sugar product assay

An Eppendorf tube was placed on ice containing 350 µl of 0.2 M acetate buffer (pH 5.2) (Appendix A2), 200 µl of 0.2% soluble potato starch and 50 µl the substrate. After incubation at 37°C for 15 min in a water both, the mixture was placed on ice before aliquots (20 µl each) were taken for sugar product determination using PC.

#### 2.4.7.3.2 α-Amylase test

This was done by selective inactivation of β-amylase during heating at a high temperature with and without $Ca^{2+}$. An Eppendorf tube was placed on ice containing 300 µl of 0.2 M acetate buffer (pH 5.2) (Appendix A2), 50 µl of 10 mM $CaCl_2$, 200 µl of 0.2% soluble potato starch and 50 µl CE. Alternatively the reaction mixture consisted of 350 µl of 0.2 M acetate buffer (pH 5.2) (Appendix A2), 200 µl of 0.2% soluble potato starch and 50 µl CE. The mixtures were first incubated at 70°C in a

water both for 5 min before they were incubated at 37°C for 15 min. After this, aliquots (20 µl each) were taken for sugar product analysis using PC.

#### 2.4.7.3.3 β-Amylase test

This was done by selective inactivation of α-amylase by lowering the pH of the incubation medium and by the use of EDTA as inhibitor. In an Eppendorf tube, the reaction mixture consisting of the following substances was incubated in a water bath at 37°C for 15 min: 350 µl of 0.1 M citric buffer (pH 3.0) (Appendix A5), 200 µl of 0.2% soluble potato starch and 50 µl CE. Alternatively, the reaction mixture consisted of 300 µl of 0.2 M acetate buffer (pH 5.2) (Appendix A2), 200 µl of 0.2% soluble potato starch, 50 µl CE and 50 µl of 40 mM EDTA in an Eppendorf tube and was incubated at 37°C for 15 min. After this, aliquots (20 µl each) were taken for sugar product analysis using PC.

### 2.4.7.4 Running PC

Sugar products from starch hydrolysis were determined by spotting the reaction mixtures onto Whatman No.1 paper (12 cm wide). Chromatographic standards used were glucose and maltose. In a sealed glass tank, PC was run 3 times each for 1 h at room temperature. A hair drier was used to dry up the paper in a fumehood between each run. Sugars on the chromatograms were visualized using the colour development reagent (see section 2.4.7.2) from a glass sprayer connected to an air source in the fumehood.

## 2.4.8. *Protein assay*

The methods used in this section followed the user's manual of GIBCOBRL Life Technologies Ltd, 1995 except otherwise indicated.

### 2.4.8.1 Preparation of protein from samples

The gentian flowers treated each with 3% (w/v) sucrose, 0.5 mM STS and $dH_2O$ (control) and the petals, sepals and reproductive parts were weighed and homogenized

in liquid $N_2$. One ml of 0.1 M sodium phosphate buffer (pH 7.0) were added to each sample in an Eppendorf tube and mixed well. After incubating for 30 min on ice the samples in Eppendorf tubes were centrifuged at 11000 rpm for 5 min at 4°C. The supernatants were stored at – 20°C.

### 2.4.8.2 Standard curve

From a stock solution (100 $mgl^{-1}$) of bovine serum albumin, Fraction V (BSA), several diluted solutions ranging from 0 to 100 $\mu g\ ml^{-1}$ were prepared. In a test tube, 1 ml of Commassie blue dye was mixed with 100 µl of a diluted protein solution (Bradford, 1976 ). The absorbance was read at $A_{595}$ nm on a spectrophotometer (Zenspec II).

### 2.4.8.3 SDS-PAGE of proteins

SDS-PAGE of gentian flower proteins was performed using the procedure as described by Laemmli (1970). 160 µl crude extract was mixed with 40 µl of a 5× SDS – PAGE sample buffers (Appendix A6) and heated in a boiling water bath for 5 min. The samples and the SDS-PAGE molecular weight standards (broad range, BIO-RAD) were loaded onto the gels BIO-RAD Mini-Protean II gel apparatus. Electrophoresis was performed at 200 V at room temperature for about 45 min. After staining with Coomassie blue for 30 min and then destaining with the destaining solution for 1 h, the gel was placed in fresh destaining solution overnight.

## 2.4.9. *Isoelectric focusing of α-amylase*

The samples (petals, sepals and reproductive parts) were homogenized in 0.2 M sodium succinate buffer (pH 5.5) (Appendix A7) containing 10 mM $CaCl_2$ (200 µl / flower) and the homogenates were heated at 70°C for 5 min, cooled in ice, and centrifuged at 10,000 rpm for 5 min. The supernatants were stored at – 20°C.

Bio-Rad Mini-Protean II Gel System was assembled following the manufacturer's instruction. For two 8 × 7 cm × 0.75 mm mini gels, the following solutions were combined in a small Erlenmeyer flask: 6.7 ml $H_2O$, 3 ml of 0.2% soluble potato starch

solution, 2 ml Solution A (Appendix A8), 48 µl ampholyte solution for pH 3.5-10 and 240 µl ampholyte solution for pH 4-6. After degassing for 15 min under vacuum, 50 µl of 10% (w/v) ammonium persulfate and 20 µl TEMED were mixed gently with the degassed acrylamide solution. About 130 ml of the catholyte (20 mM sodium hydroxide) were placed in the upper buffer chamber, and 250 ml anolyte (10 mM phosphoric acid) were placed in the lower buffer chamber.

Each enzyme sample was mixed with an equal volume of 2 × sample buffer (Appendix A9) (eg. 15 µl : 15 µl) and centrifuged at 10,000 rpm (Eppendorf centrifuge) for 5 min before the cleared samples were loaded onto the gels. Isoelectric focusing gels were run at room temperature at 200 V for 1.5 h and then 400 V for 1.5 h (constant voltages). The gels were removed from the mini-gel apparatus, and then stained with the iodine reagent (Appendixes A3 and A4) for 10 min in the dark.

## 2.5. ACC oxidase gene expression - A preliminary study

### 2.5.1. *Plant material*

Fresh *in vitro* flowers at different stages, e.g. blue buds, opened flowers and wilted flowers, were collected in the Plant Biotechnology Laboratory, Department of Plant and Microbial Sciences, University of Canterbury, Christchurch, New Zealand.

### 2.5.2. *RNA preparation*

The methods used in this section followed the user's manual of GIBCOBRL Life Technologies Ltd. Except otherwise indicated.

#### 2.5.2.1 RNA isolation

Fresh petals (80-100mg) of *in vitro* flowers were homogenized in 1 ml of TRIZOL Reagent (GIBCOBRL Life Technologies, NY, USA) using a mortar and pestle. The homogenized tissue samples were transferred to a 1.5 ml Eppendorf tube. Following

incubation of the homogenized samples for 5 min at room temperature, 0.2 ml chloroform was added to the tube. The tube was capped securely and shacked vigorously by hand for 15 seconds and incubated at room temperature for 2 to 3 min. Following centrifugation at 11,000 rpm for 15 min at 4°C, the mixture was separated into a lower red, phenol-chloroform phase, a white organic inter-phase, and a purple upper aqueous phase. RNA was partitioned exclusively in the aqueous phase. The RNA was precipitated from the aqueous phase by mixing with 0.5 ml of isopropyl alcohol. After incubation at room temperature for 10 minutes, the mixture was centrifuged at 11,000 rpm for 10 min at 4°C. After removal of the supernatant, the RNA pellet was washed twice with 1 ml of 75% ethanol by vortexing and centrifuging at 7,500 rpm for 5 min at 4°C. Following removal of the ethanol, the RNA pellet was dried under vacuum for 15 min, dissolved in 50 µl of RNase-free water (Appendix C1) and incubated for 10 min at 55 to 60°C in a water bath. The RNA was stored at – 80°C until use.

### 2.5.2.2 Quantification of isolated total RNA

Total RNA was successfully isolated from petals of *in vitro G. triflora* flowers. These included flowers treated with 3% sucrose or 0.5mM STS plus control (water pulsed). The RNAs were diluted with RNase-free $dH_2O$ by 50 or 100 folds before their amounts were quantified using a spectrophotometer (LKB Biochrom Ultrospec® Plus 4054). A total of 300 µl of diluted RNA sample was enough for one measurement and 3 replicates for each sample were tested. Partially dissolved RNA samples had an $A_{260/280}$ ratio < 1.6 (according to the user's manual of GIBCOBRL Life Technologies Ltd., 1995). Total RNA concentration was calculated using the data obtained from the spectrophotometer according to following formula:

$$C_{RNA} = f \cdot u \cdot df$$

(Dr. Leung, Universtiy of Canterbury, Christchurch, New Zealand, personal communation)

Where

$C_{RNA}$ is the concentration of RNA ($\mu g\ ml^{-1}$)

$f$ is the coefficient of RNA concentration at $A_{260}$

1 unit of RNA at $A_{260}$ = 40 $\mu g\ ml^{-1}$ (according to the user's manual of GIBCOBRL Life Technologies Ltd., 1995)

u is the data (unit) read at $A_{260}$

df is the fold to the dilution of the original RNA solution

therefore

$C_{RNA}$ Control = $f \cdot u \cdot df = 40 \times 0.2 \times 50 = 400\ \mu g\ ml^{-1} = 0.4\ \mu g\ \mu l^{-1}$

$C_{RNA}$ Sucrose = $f \cdot u \cdot df = 40 \times 0.094 \times 100 = 376\ \mu g\ ml^{-1} = 0.4\ \mu g\ \mu l^{-1}$

$C_{RNA}$ STS = $f \cdot u \cdot df = 40 \times 0.153 \times 100 = 610.6\ \mu g\ ml^{-1} = 0.6\ \mu g\ \mu l^{-1}$

## 2.5.3. *Reverse transcription to generate cDNA templates*

To generate cDNA templates, reverse transcription was performed in combination with PCR.

### 2.5.3.1 Primer designing

The primers for amplification of a conserved domain of ACC oxidases in several plants were designed by Dr. Leung (Universtiy of Canterbury, Christchurch, New Zealand, personal communation) as follows:

Primer 1: downstream primer AO1

5'-CC(G/A/T/G)CC(C/T/T/G)TG(T/G)CC(A/G/A/A)AA(C/T)CC(G/G/G/G)GA-3'

Primer 2: upstream primer AO2

5'-TT(G/C/T/T)CC(C/A/A/A)TG(T/C)CC(T/T/G/G)AA(C/T)CC(C/C/T/T)GA-3'

The designed primers were manufactured by GIBCOBRL Life Technologies LTD, Auckland, New Zealand.

### 2.5.3.2 Optimization of RNA concentration in RT-PCR

Titan™ One Tube RT-PCR System (Roche Diagnostics GmbH, Mannheim, Germany) was utilized in this study. For preparation of master mixes, the reaction components were set up on ice as shown in Table 2-1 and Table 2-2 for Master mix I and Master mix II in 3 separate, nuclease free 0.2ml-thin-wall-PCR-tubes (Salmond Smith Biolab Ltd, Auckland, New Zealand):

**Table 2-1 Master Mix I for RT-PCR**

| Components | Tube 1 (control) Volume (µl) | Tube 2 (sample) Volume (µl) | Final concentration in the RT-PCR |
|---|---|---|---|
| dNTP (10mM) | 1 | 1 | 0.2 mM |
| downstream primer AO1(20µM) | 1.3 | 1.3 | 0.4 µM |
| upstream primer AO2 (20µM) | 1.3 | 1.3 | 0.4 µM |
| template RNA | 0 | 1* | 0.4 µg* |
| DTT-solution (100µM) | 2.5 | 2.5 | 5 mM |
| sterile $dH_2O$ | 18.9 | 17.9 | |
| **Total** | **25** | **25** | |

*1µl, 2µl and 3µl of template RNA were used, each containing 0.4µg, 0.8µg and 1.2µg total RNA in samples, respectively. This was designed to test the effect of the amount of RNA on the efficiency of RT-PCR. Sterile $dH_2O$ was reduced to 16.9 µl and 15.9 µl from 17.9 µl, respectively, according to the RNA volume used.

**Table 2-2 Master Mix II for RT-PCR**

| Components | Tube 3 Volume (µl) | Final concentration in the RT-PCR |
|---|---|---|
| 5× RT-PCR buffer with $Mg^{2+}$ | 20 | 1.5 mM $MgCl_2$ |
| Enzyme mix | 2 | AMV and Expand High Fidelity PCR-System |
| sterile $dH_2O$ | 28 | |
| **Total** | **50** | |

From tube 3, 25 µl of the mixture were taken and added to tube 1 or tube 2 in the master mix I (Table 2-1). The tubes were centrifuged briefly to collect the sample at the bottom of the tubes and then 30 µl of mineral oil was overlaid in each tube. Program TED I (Appendix D1) was run for the thermal cycling on a Programmable Thermal Controller (PTC-100, MJ Research, Inc.).

### 2.5.3.3 Testing RT-PCR results by electrophoresis through agarose gels

BIO-RAD MINI SUB™ DNA gel electrophoresis apparatus was used for running agarose gel electrophoresis. TAE buffer was prepared as a 50× stock solution (Appendix C2). To make a 1% (w/v) gel in 30 ml of 1 × TAE buffer, 0.33g agarose (BIO-RAD) were mixed with 30 ml of the buffer. The agarose was dissolved after heating in a microwave oven for about 1 min. Five µl of DNA molecular weight marker (Appendix C3) and 10 µl sample mixed with 5 µl DNA loading buffer (Type III) (Appendix C4) were loaded into the slots of the submerged gel. The agarose gel electrophoresis was run at 80V for 1 hour. After this, the gel was stained with ethidium bromide (0.5 µg $ml^{-1}$) for 10 min at room temperature, washed in running

water for 15 min and examined under UV light. The gels were photographed using a camera (Electrophoresis Documentation and analysis System 120, Kodak Digital Science) and an electronic UV transilluminator (ULTRA · LUM). The molecular weight of newly synthesised cDNA was calculated according to the equation displayed on the standard curve,

$$y = 92.165x - 1465.2 = 9216.5 \times 26 - 1465.2 = 931 \text{ bp } (0.931 \text{ kb})$$

(This equation is obtained from the molecular weight standard curve made using Scientific Graphing Software "SigmaPlot®")

### 2.5.4. *Recovery of DNA product from low-melting-temperature agarose*

In this study, low-melting-temperature (LMT) agarose was used for recovery of DNA fragment produced from RT-PCR.

LMT agarose at 0.7% (w/w) was dissolved by heating in the electrophoresis buffer (1 × TAE buffer, Appendix C5) at 70°C. After cooling to 37°C, the agarose solution was poured and kept at 4°C to ensure that the gel was set properly. The sample DNAs, 10 µl each mixed with 5 µl loading buffer were loaded onto the agarose gel and electrophoresis was carried out at 4°C for 70 min, at 80V. After this, the gel was stained in 0.5µg $ml^{-1}$ of ethidium bromide solution for 10 min, rinsed in tap water for 15 min, and the DNA segment on the gel was cut under UV light and placed in an Eppendorf tube. Five volumes of 20 mM Tris·HCl (pH8.0) and 1 mM EDTA were added. The gel was melted by heating at 65°C for 5 min in a water bath. At room temperature, the melted gel slices were extracted with an equal volume of phenol (0.2 ml). The aqueous phase was recovered by centrifugation at 20°C for 5 min, and then re-extracted with phenol/chloroform followed by chloroform. The DNA was recovered by precipitation with 0.4 ml of absolute ethanol followed by 0.4 ml of 70% ethanol. The DNA was dried in a vacuum for 15 min before it was dissolved in 20 µl sterilized $dH_2O$.

Yield of the DNA recovered from LMT agarose was determined using a spectrophotometer (LKB Biochrom Ultrospec® Plus 4054) to measure absorbance at $A_{260}$. The concentration of recovered cDNA was then calculated according to the readings and the following formula. One $A_{260}$ unit equals 50μg of double-stranded DNA/ml (according to the user's manual of GIBCOBRL Life Technologies Ltd.,1995).

$$C_{DNA} = f.u.df$$

(Dr. Leung, Universtiy of Canterbury, Christchurch, New Zealand, personal communation)

where

$C_{DNA}$ is the concentration of DNA ($\mu g\ ml^{-1}$)

$f$ is the coefficient of DNA concentration at $A_{260}$

1 unit of DNA at $A_{260}$ = 50 $\mu g\ ml^{-1}$

u is the data (unit) read at $A_{260}$

df is the fold to the dilution of the original DNA solution

therefore

$C_{DNA} = f \cdot u \cdot df = 50 \times 0.021 \times 100 = 105\ \mu g\ ml^{-1} = 0.105\ \mu g\ \mu l^{-1}$

## 2.5.5. *The amplification of cDNA directly from RT-PCR products*

### 2.5.5.1 Procedure of re-amplification using RT-PCR product as the template

Different concentrations of the RT-PCR product was tested to further optimize the PCR reaction. The volumes used were listed in Table 2-4, where 1, 5 or 10 μl of the template was tested and the volume of sterile $dH_2O$ was reduced to 39.6 μl or 34.6 μl from 43.6 μl according to the increasing volume of the templates.

**Table 2-4 Optimization of the concentration of RT-PCR product in DNA amplification using PCR**

| Components | Volume (µl) | Final concentration in the PCR |
|---|---|---|
| dNTP (10mM) | 1 | 0.2 mM |
| downstream primer AO1 (20µM) | 1.3 | 0.4 µM |
| upstream primer AO2 (20µM) | 1.3 | 0.4 µM |
| template (cDNA) | 1 (5 or 10) | ≈1µg (5 or 10µg) |
| Taq (5 units $\mu l^{-1}$) | 1 | 0.42 U |
| Buffer (10x) | 6 | 1 x |
| $MgCl_2$ (1.5mM) | 4.8 | 7.2 mM |
| sterile $dH_2O$ | 43.6 (39.6 or 34.6) | |
| **Total** | **60** | |

## 2.5.6. *DIG DNA labelling and detection*

For labelling of the RT-PCR product, digoxigenin (DIG) was coupled to dUTP via an alkali-labile ester-bond. The use of the alkali-labile form of DIG-11-dUTP enables easier and more efficient stripping of blots for re-hybridization experiments with a second DIG-labelled probe. The DNA probes, labelled with DIG-11-dUTP, were denatured by incubation in a boiling water bath.

### 2.5.6.1 DNA labelling

DIG-labelled DNA probes were generated enzymatically according to the method of random primed labelling which is based on the hybridization of random oligonucleotides to the denatured DNA template. The complementary DNA strand was synthesized using the Klenow enzyme (Roche Diagnostics GmbH, Mannheim, Germany) that used the 3'OH termini of the random oligonucleotides as primers and a

mixture of deoxyribonucleosides containing DIG-11-dUTP resulting in incorporation of digoxigenin into the newly synthesized DNA. To test the labelling efficiency, two types of enzyme were tried; these were Klenow enzyme and Taq polymerase. DIG DNA labelling reactions were performed by mixing the components shown in Table 2-5.

**Table 2-5 DIG-labelling DNA by PCR**

| Components | Volume (μl) | Final concentration in the PCR |
|---|---|---|
| dNTP* (1mM) | 5 | 0.42μM |
| dNTP (1mM) | 5 | 0.42μM |
| Downstream primer AO1 (20μM) | 1.3 | 0.4 μM |
| Upstream primer AO2 (20μM) | 1.3 | 0.4 μM |
| DNA Template | 2 | ≈ 2 μg |
| Klenow (2U $\mu l^{-1}$) or Taq (5U $\mu l^{-1}$) | 1 | Klenow 0.07 U; Taq 0.42U |
| Buffer (10×) | 6 | 1 × |
| $MgCl_2$ (1.5 mM) | 4.8 | 7.2 mM |
| $ddH_2O$ | 33.6 | |
| **Total** | **60** | |

*Contains dUTP

DIG DNA labelling was via standard PCR procedure. The programme TED II was performed for this experiment (Appendix D2).

### 2.5.6.2 Quantification of labelling efficiency

The yield of DIG-labelled DNA was estimated in comparison to a DIG-labelled control-DNA in a dot blot followed by direct immunodetection with the colour substrate NBT/BCIP.

DIG-labelled control-DNA was diluted 1:5 using sterile $dH_2O$ to a concentration of 1 $\mu g\ ml^{-1}$. After preparing the dilution series of DIG-labelled control-DNA, a series of the dilutions ranging from 0.01 pg to 10 pg were spotted (1µl/spot) onto a dry nylon membrane (positively charged, Boehringer Mannheim). After drying up at room temperature, the membrane was cross-linked by UV light using an UV transilluminator (Mineralight®, UVSL-25, Watson Victor LTD, Australia) for 3 min, then incubated in blocking solution (1×) (Appendix C6) at room temperature for 30 min and in antibody solution (Appendix C7) for 30 min at room temperature. The membrane was washed 2 × 15 min in washing buffer (Appendix C8) and equilibrated for 5 min in detection buffer (Appendix C9) at room temperature. After incubating in freshly prepared colour substrate solution (Appendix C10) for 5 min (no shaking during colour development, this is very important), the membrane was placed into a petri dish. The petri dish was put in darkness for 24 hours at room temperature. The reaction was stopped by washing the membrane in water for 5 min. Spot intensities of the control and experimental dilutions were compared to estimate the concentration of the DIG-labelled probe DNA. Finally, the results were documented by scanning using a scanner (Nikon Scantouch).

## 2.5.7. *RNA Dot Blotting*

### 2.5.7.1 Sample RNA treatment

Gentian petal RNAs were diluted to > 0.2 $\mu g\ \mu l^{-1}$ in RNA dilution buffer (Appendix C11) before 1µl of each sample was spotted onto a dry nylon membrane (positively changed, Boehringer Mannherim) using a micropipettor. The RNAs were fixed to the membrane by UV crosslinking for 3 min using an UV transilluminator (Mineralight®, UVSL-25, Watson Victor LTD, Australia).

### 2.5.7.2 Hybridization with DNA probe

The blot (≈ 1 $cm^2$) was placed in an Eppendorf tube containing 200 µl pre-hybridization solution (Appendix C12) (20 ml pre-hybridization solution per 100 $cm^2$ of membrane surface area) and then pre-hybridized at 50°C in a water bath for 2 hours. After heat denaturing the DIG-labelled probe in a boiling water bath for 10 min, the probe (100 ng $ml^{-1}$) was diluted to 25 ng $ml^{-1}$ in the pre-hybridization solution. The RNA blot was immersed in the hybridization solution containing the DIG-labelled probe and hybridized overnight at 50°C. At the end of the hybridization, the membrane was washed twice, 15 min per wash, in 2 × wash solution (Appendix C13) at room temperature and then washed twice in 0.5 × wash solution (Appendix C14) at 68°C, 15 min per wash.

### 2.5.7.3 Immunological detection

After hybridization and post-hybridization washes, the blot was equilibrated in washing buffer (Appendix C15) for 1 min, incubated in blocking solution (Appendix C6) for 1 h, and then in antibody solution (Appendix C7) for 30 min. After this, the blot was gently washed twice, 15 min per wash, in washing buffer (Appendix C8), equilibrated in detection buffer (Appendix C9) for 2 min, and was incubated in colour substrate solution (Appendix C10) in a covered container in a dark room. The formation of a coloured precipitate started within a few min, and completed after 12 hours. Once the desired spots were detected, the membrane was washed with $H_2O$ to prevent over-development of the colour in the background. The results were documented after scanning using a scanner (Nikon Scantouch).

# Chapter 3. Results

## 3.1. *In vitro* flower system

### 3.1.1. *Flowering gradient*

Preliminary tests indicated that it is easy to initiated vegetative shoots (from pre-existing axillary buds) and flowers from nodal explants obtained near apices. Nodal explants were taken sequentially starting from the apices (first to fifth node) to investigate the influence of explant source / position on *in vitro* shoot and flower formation. Flowering occurred more on the explants of the first, second and third nodal segments than on the others (Figure 3-1). In contrast, a high level of shoot formation occurred on the first three nodal segments and was only slightly lower in the fourth and fifth ones (Figure 3-1).

### 3.1.2. *Effect of $GA_3$*

Shoot induction of *G. triflora* was greatly stimulated as multiple small fine shoots grown from the leaf axils of each nodal segment after 5 weeks of culture on the double strength WPM medium containing as little as 0.01 mg $l^{-1}$ $GA_3$. However, the replacement of BA with $GA_3$ at the concentrations tested did not produce flowers. No flowering was observed after 12 weeks of culture (Figure 3-2).

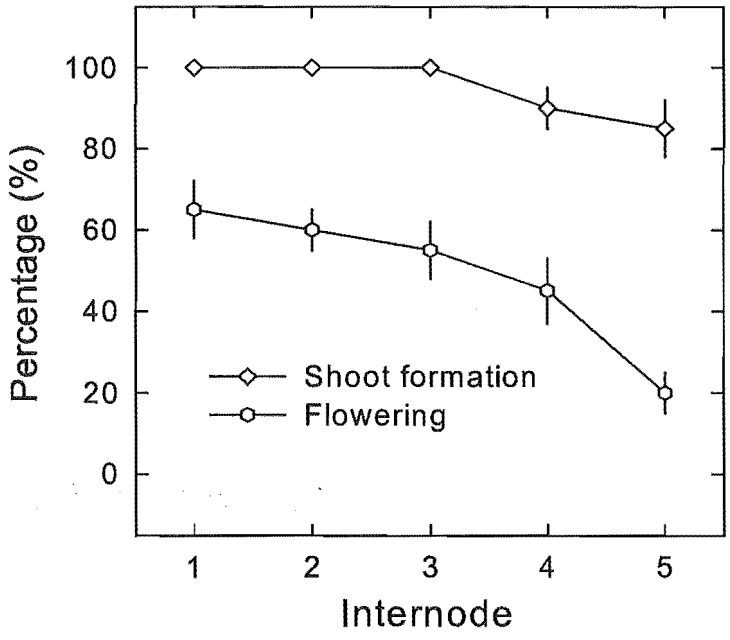


**Figure 3-1** ***In vitro*** **shoot and flower formation of** ***G. triflora***

*In vitro* shoot and flower formation as influenced by position of shoot nodal segments (1st to 5th, starting from the apex) of *G. triflora* after 5 and 12 weeks of culture, respectively. Each value is the means ± SE of the combined data from 3 repeat experiments (n =10 nodal segments in each experiment).

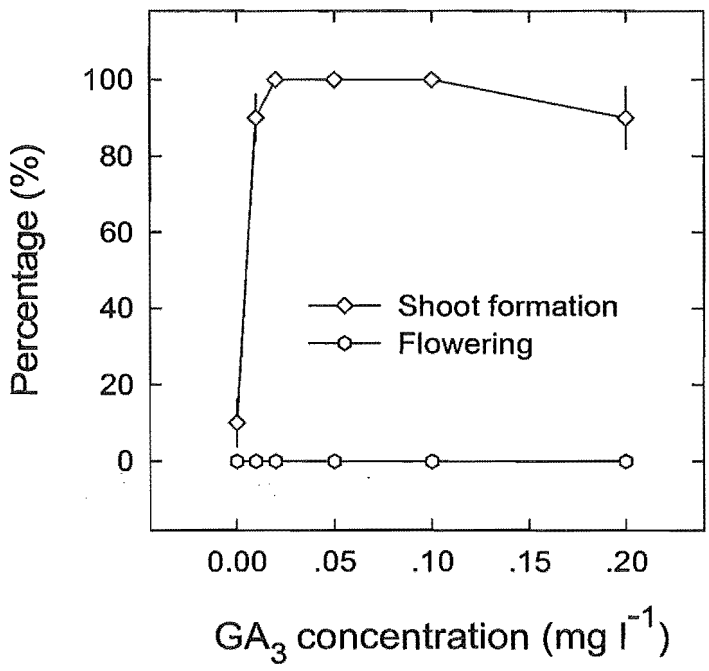


**Figure 3-2 Effect of $GA_3$ on shoot and flower formation**

Effect of different concentrations of $GA_3$ on shoot and flower formation from nodal segments of *G. triflora* cultured on double strength WPM medium containing 2% sucrose. Shoot and flower numbers were counted after 5 and 12 weeks of culture, respectively. Each value is the means ± SE of the combined data from 3 repeat experiments (n =10 nodal segments in each experiment).

### 3.1.3. *Effect of pre-incubation in the dark*

Shoot and flower formation in nodal explants was very poor in the absence of sucrose in the medium at the PPFD of 60 μmol $m^{-2}$ $s^{-1}$ (Figure 3-3 and Figure 3-4). Addition of as little as 1% of sucrose to the medium greatly improved shoot formation (Figure 3-3). The percentage of shoot-forming response after 5 weeks in the dark or light (PPFD at 60 μmol $m^{-2}$ $s^{-1}$) was different. The results revealed that it was easier to form shoot in the dark than in the light condition. In the dark, more shoots were produced but they were long fine etiolated shoots (Figure 3-5) contrasting with those normal green shoots formed on the explants in light. When the shoots initiated in the dark were transferred to the light condition for another 7 weeks, no flower formation was observed (Figure 3-4). However, those shoots initiated and cultured in the light condition for the entire 12 weeks were capable of forming flowers, the best response (about 70% forming flowers) being that occurring in the medium containing 3% sucrose.

### 3.1.4. *Interaction between sucrose and light intensity*

The influence of sucrose concentration on shoot formation was also evident at 2 other PPFDs tested, i.e. 30 and 120 μmol $m^{-2}$ $s^{-1}$ (Figure 3-6). While 2% sucrose appeared to be optimal for shoot formation at all 3 PPFDs, the response was only 60% at 120 μmol $m^{-2}$ $s^{-1}$ compared to 100% at the other 2 PPFDs.

The sucrose concentration in the medium that brought about optimal flowering response varies with the PPFDs in which the cultures were grown (Figure 3-7). The best flowering response at low, medium and high PPFD was 50, 70 and 40% respectively when the sucrose concentration was 4, 3 and 2% respectively.

### 3.1.5. *Interaction between BA and light intensity*

The effect of several cytokinins, namely BA, kinetin, zeatin and 2iP, on flowering of *G. triflora in vitro* was investigated in a preliminary experiment. It appears that all the cytokinins tested were capable of triggering *in vitro* shoot and flower formation to different extents but BA gave the best response (data not shown).

At low or medium PPFD (30 or 60 μmol $m^{-2}$ $s^{-1}$), most of the nodal explants responded to 0.2 to 1.0 mg $l^{-1}$ BA in producing 1-3 shoots from the leaf axils after 5 weeks of culture (Figure 3-8). By contrast, most BA concentrations tested resulted in 40% or less shooting response at the high PPFD (120 μmol $m^{-2}$ $s^{-1}$).

Upon further culture for another 7 weeks, the cultures kept in the medium light intensity performed better than those in the high light intensity as far as flowering response is concerned (Figure 3-9). In both light conditions, the largest percent (70 and 50% respectively) of the cultures formed flowers when the concentration of BA in the medium was 0.2 mg $l^{-1}$. In contrast, at the low light intensity, at least 0.5 mg $l^{-1}$ BA in the medium was required to bring about the maximal response, i.e. 50% of the cultures forming flowers (Figure 3-9). Most flowers were normal and complete but some were small and malformed with undeveloped stigma.

### 3.1.6. *Interaction between pH and light intensity*

In general, at the 3 levels of PPFDs the best shoot-forming response occurred when the pH of the medium was 5.7 (Figure 3-10). The influence of pH of the medium on shoot formation was similar at the low and medium PPFDs. In contrast, the shoot-forming response at the high PPFD was more adversely affected, particularly at pH of the medium greater than 5.7.

Flower formation was maximal (about 60%) at pH 4.7 and far greater when the *in vitro* shoots were grown in the medium rather than the low or high PPFD (Figure 3-11). In general, at the range of pH values tested, flower formation at both the low and high PPFD was rather poor, i.e. often below 25%.

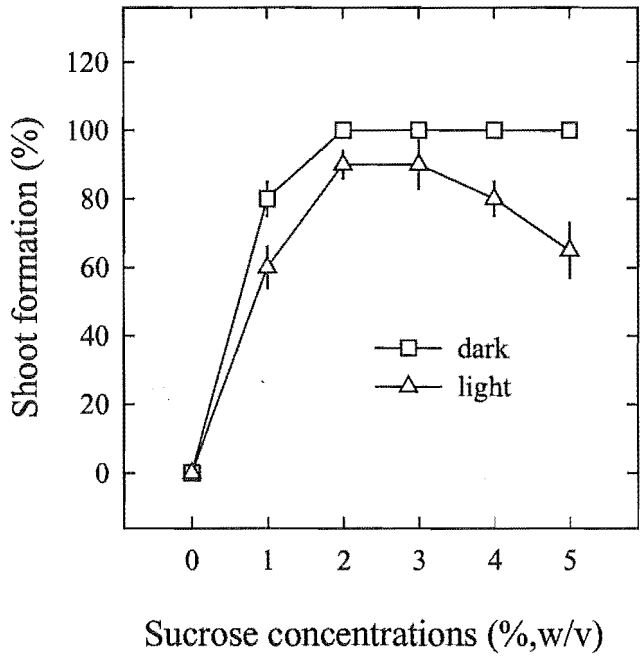


**Figure 3-3 Effects of light and darkness on shoot formation**

Effects of light (12 weeks at PPFD of 60 μmol $m^{-2}$ $s^{-1}$) or darkness (5 weeks in dark before transfer to the light condition for another 7 weeks) on shoot formation from nodal segments of *G. triflora* shoots cultured on double strength WPM media containing different sucrose concentrations and 0.5 mg $l^{-1}$ BA. All data were obtained at the end of 5 weeks of culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

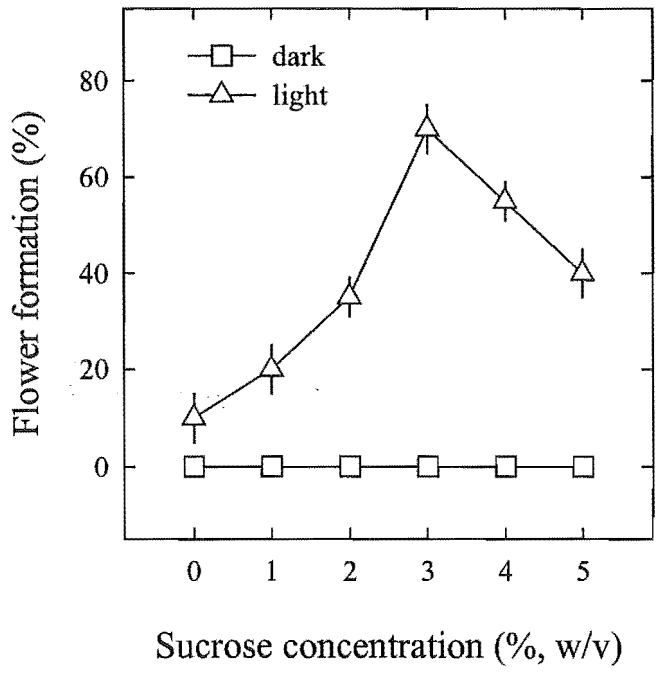


**Figure 3-4 Effects of light and darkness on flower formation**

Effects of light (12 weeks at PPFD of 60 μmol $m^{-2}$ $s^{-1}$) or darkness (5 weeks in dark before transfer to the light condition for another 7 weeks) on flower formation from nodal segments of *G. triflora* shoots on double strength WPM media containing different sucrose concentrations and 0.5 mg $l^{-1}$ BA. All data were obtained at the end of 12 weeks of culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

**Figure 3-5** ***In vitro*** **shoots grown in dark (left) and normal light condition (right) for 5 weeks**

Nodal segments of *G. triflora* were grown on double strength WPM media containing different 3% sucrose and 0.5 mg $l^{-1}$ BA in darkness for 5 weeks. These shoots are long, thin and colourless with underdeveloped colourless leaflets (left panel) comparing to the shoot produced in normal light condition (right panel).

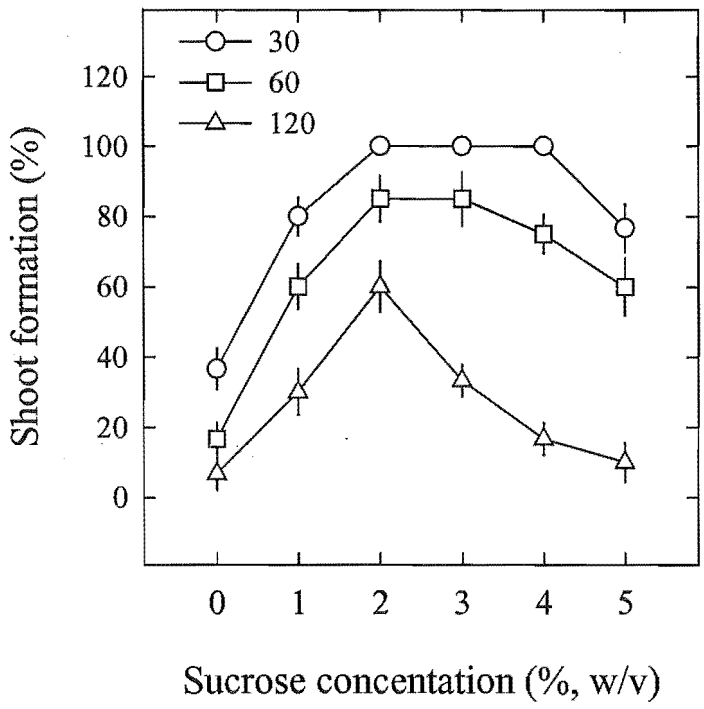


**Figure 3-6 Effect of PPFD and sucrose concentration on *in vitro* shoot formation from nodal segments of *G. triflora***

The medium was double-strength WPM containing 0.5mg $l^{-1}$ BA and the appropriate level of sucrose. Shoot numbers were counted after 5 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

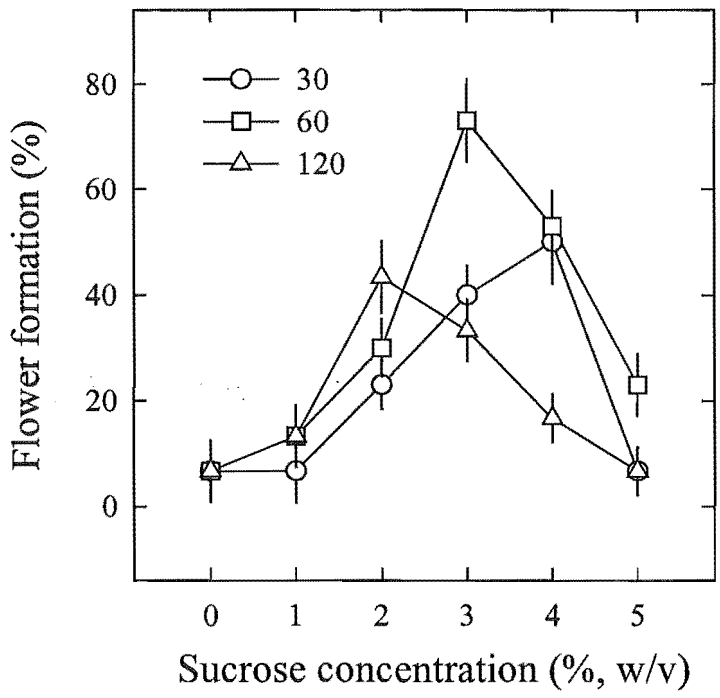


**Figure 3-7 Effect of PPFD and sucrose concentration on *in vitro* flower formation from nodal segments of *G. triflora***

The medium was double-strength WPM containing 0.5 mg $l^{-1}$ BA and the appropriate level of sucrose. Flower numbers were counted after 12 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

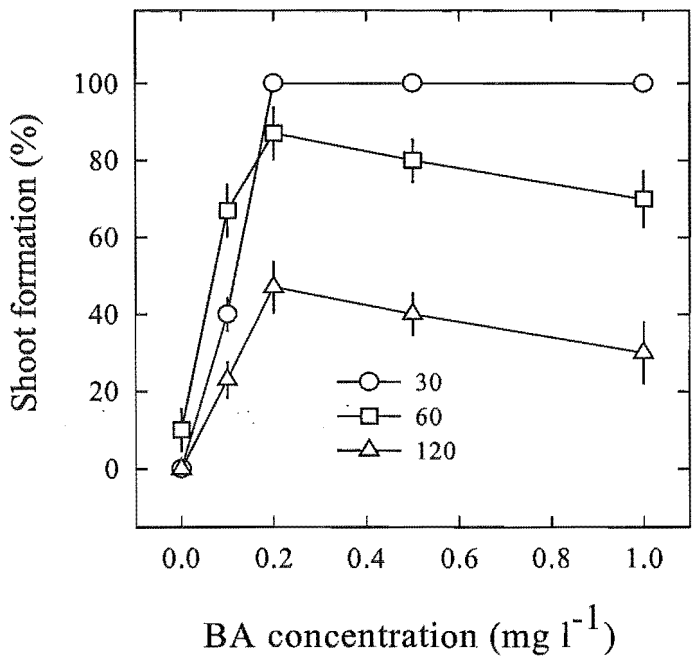


**Figure 3-8 Effect of PPFD and BA concentration on shoot formation from nodal segments of *G. triflora***

The medium was double strength WPM containing 3 % sucrose and the appropriate level of BA. Shoot numbers were counted after 5 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

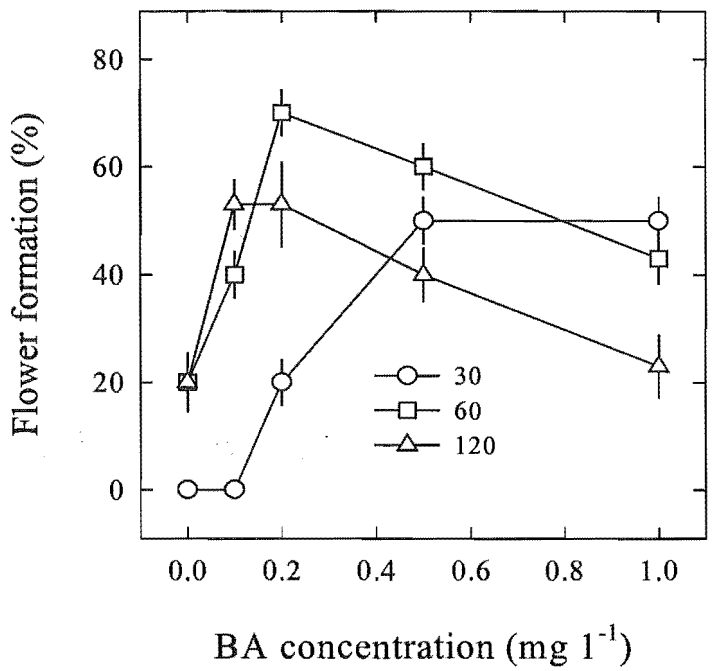


**Figure 3-9 Effect of PPFD and BA concentration on flower formation from nodal segments of *G. triflora***

The medium was double strength WPM containing 3 % sucrose and the appropriate level of BA. Flower numbers were counted after 12 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

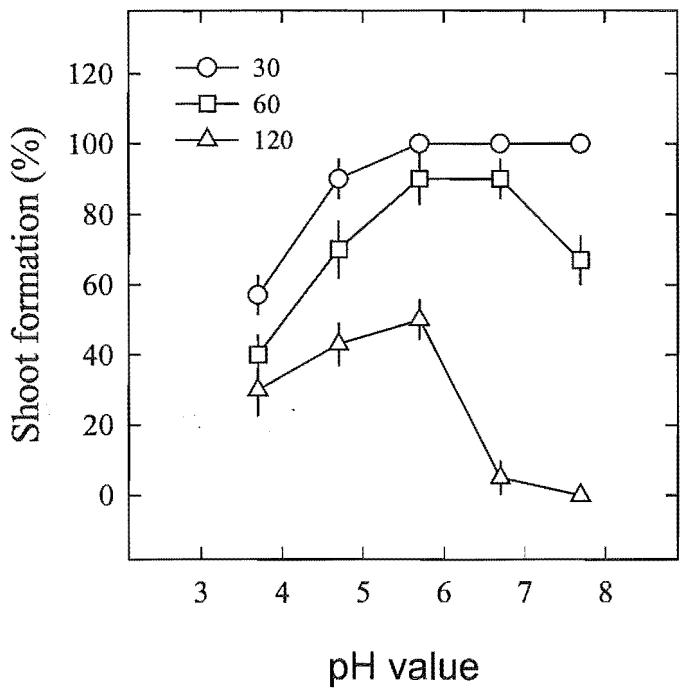


**Figure 3-10 Effect of PPFD and pH on shoot formation from nodal segments of *G. triflora* shoots**

The double strength of WPM containing 0.5 mg $l^{-1}$ BA and 3% (w/v) sucrose was adjusted to the different pH values. Shoot numbers were counted after 5 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

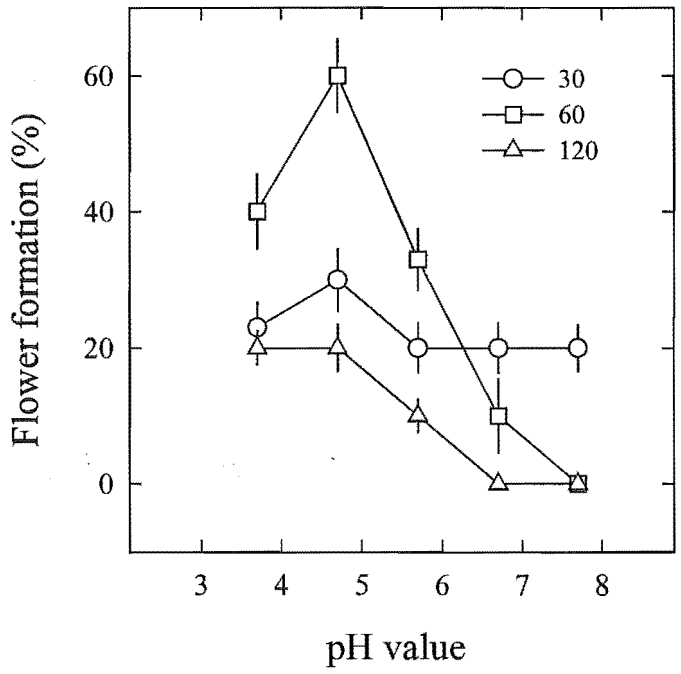


**Figure 3-11 Effect of PPFD and pH on flower formation from nodal segments of *G. triflora* shoots**

The double strength of WPM medium containing 0.5 mg $l^{-1}$ BA and 3% (w/v) sucrose was adjusted to the different pH values. Flower numbers were counted after 12 weeks in culture. Each value is the means ± SE of the combined data from 3 experiments (n =10 nodal segments in each experiment).

## 3.1.7. *Comparison of in vitro and in vivo flowers*

*In vitro* flowers were produced from cultured nodal segments of *G. triflora* in 250-ml polycarbonate culture vessels containing double-strength WPM medium with 3% sucrose (w/v), 0.2 mg $l^{-1}$ BA and pH 4.7 under PPFD at 60 μmol $m^{-2}$ $s^{-1}$ (Figure 3-12).

### 3.1.7.1 Morphological similarities

The *in vitro* flowers were compared, at anthesis stage, with those produced by plants grown by a local cut-flower gentian grower (herein referred to as "*in vivo*" flowers) in terms of flower size, shape and colour (Figure 3-13 and Table 3-1). The flowers formed under *in vitro* and *in vivo* conditions were bell-shaped with semi-opened corolla which is typical of Gentianaceae family (Kohlein, 1991). The corolla appeared light blue in *in vitro* flowers and deep blue in *in vivo* ones. Another notable difference is that the corolla length of *in vitro* flowers was smaller than that of *in vivo* ones. Most *in vitro* flowers had the same number of easily recognizable reproductive parts (i.e. the stigma and 5 anthers) although they were smaller than those of the *in vivo* flowers were. About 40% of both types of flowers had stigma that was not fully developed, i.e. it was not split open and resembled a pointed needle. Only the flowers with fully developed or split open stigma could receive pollen grains on the surface of the lip-like structure.

### 3.1.7.2 SEM examination of pollen grain

The pollen grains from *in vitro* and *in vivo* flowers appeared to be very similar in size as observed using the SEM (Figures 3-14 to 3-19; also see Table 3-1).

**Figure 3-12** ***In vitro*** **flowers of** ***G. triflora***

*In vitro* flowers were produced in 250-ml polycarbonate culture vessels containing double-strength WPM medium with 3% sucrose (w/v), 0.2 mg $l^{-1}$ BA and pH 4.7 under PPFD at 60 μmol $m^{-2}$ $s^{-1}$.

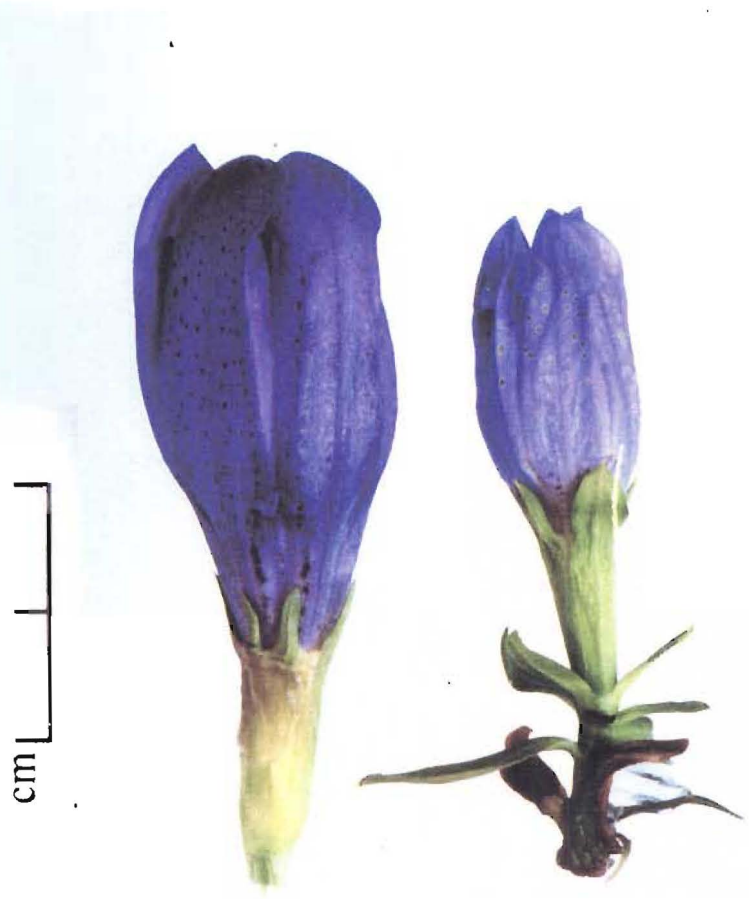


**Figure 3-13 Comparison of *in vitro* and *in vivo* flower of *Gentiana triflora* Pall. var. *axillariflora* Akita Blue**

A comparison of the *in vitro* and *in vivo* flowers (right and left panels, respectively). The flowers formed under *in vitro* or *in vivo* conditions were bell-shaped that are typical of Gentianaceae family. The corolla appeared light blue in *in vitro* and deep blue in *in vivo* flowers.

**Table 3-1 Comparison of *in vitro* and *in vivo* floral organs of *Gentiana triflora* Pall. var. *axillarflora*[1]**

| Flower source | Flower shape | Flower colour | Corolla length[2] (mm) | Anther number | Fully developed stigma[2] (%) | Pollen length ($\mu$m) | Pollen viability (%) | Pollen germination (%) | Seed length (mm) | Length of seed-like structure (mm) |
|---|---|---|---|---|---|---|---|---|---|---|
| *In vitro* | Bell-shaped | Light blue | 28.8 ± 0.62a | 4.6 ± 0.08a | 54 ± 2.98a | 39.7 ± 0.65a | 90 ± 2.25a | 10.7 ± 3.42a | – | 1.4 ± 0.03a |
| *In vivo* | Bell-shaped | Deep blue | 39.8 ± 0.55b | 5.0 ± 0.03b | 60 ± 1.88a | 40.8 ± 0.68a | 94 ± 1.27a | 33.7 ± 2.65b | 2.4 ± 0.04 | 1.7 ± 0.04b |

[1] Values are means of 50 replicates ± SE except those of pollen viability and pollen germination in which 3 replicates were used. Data marked by same letter in a column are not significantly different (Tukey's Test, $P < 0.05$).

[2] At anthesis

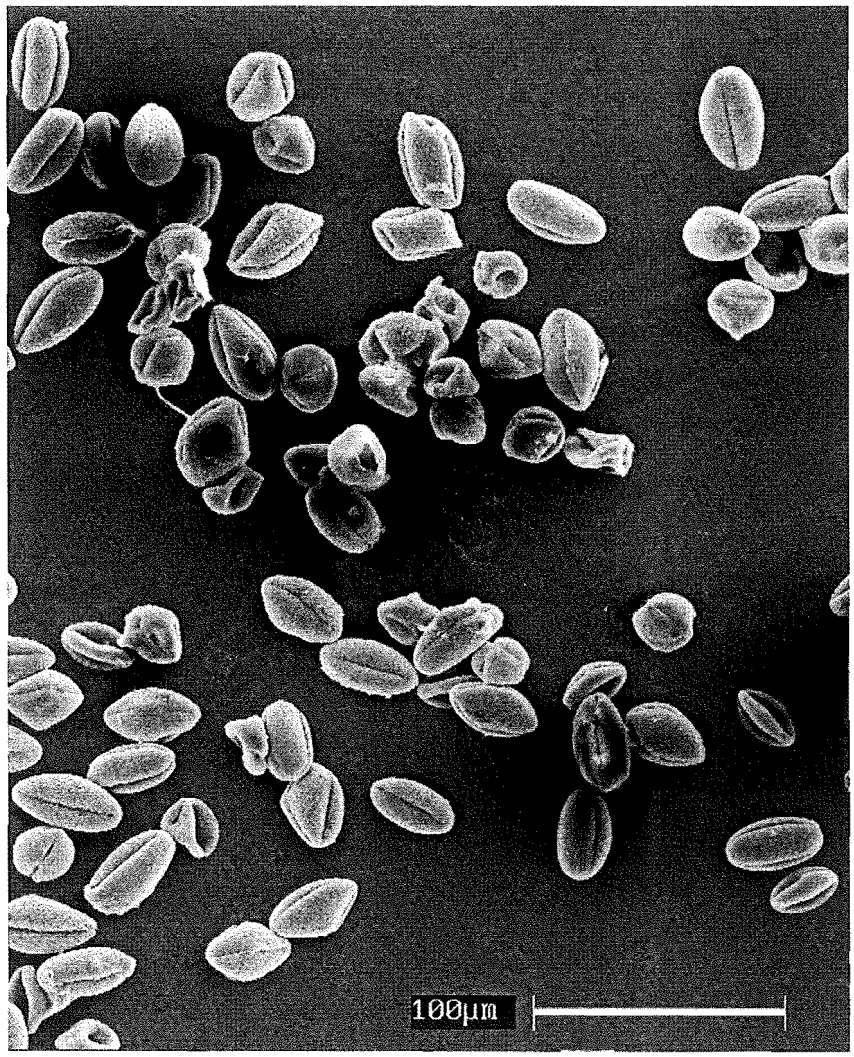


**Figure 3-14 Pollen grains from *in vitro* flower of *G. triflora***

*In vitro* pollen grains of *G. triflora* were observed through SEM at a low amplification.

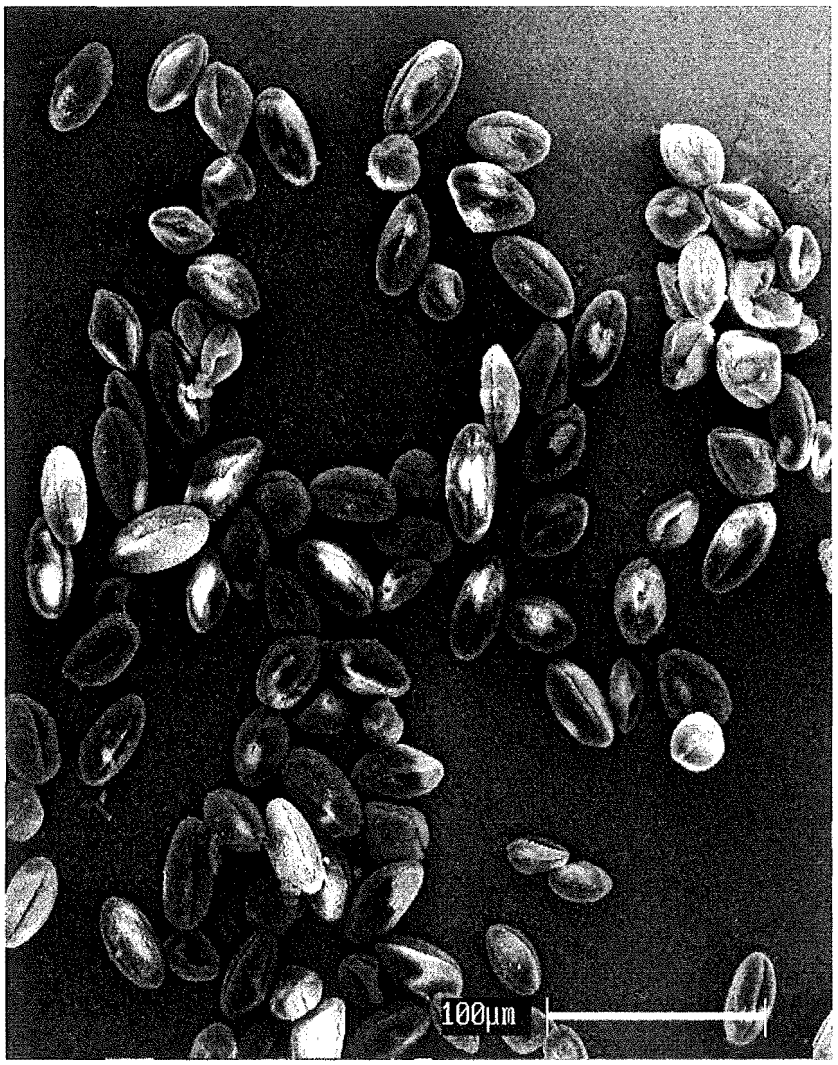


**Figure 3-15 Pollen grains from *in vivo* flower of *G. triflora***

*In vivo* pollen grains of *G. triflora* were observed through SEM at a low amplification.

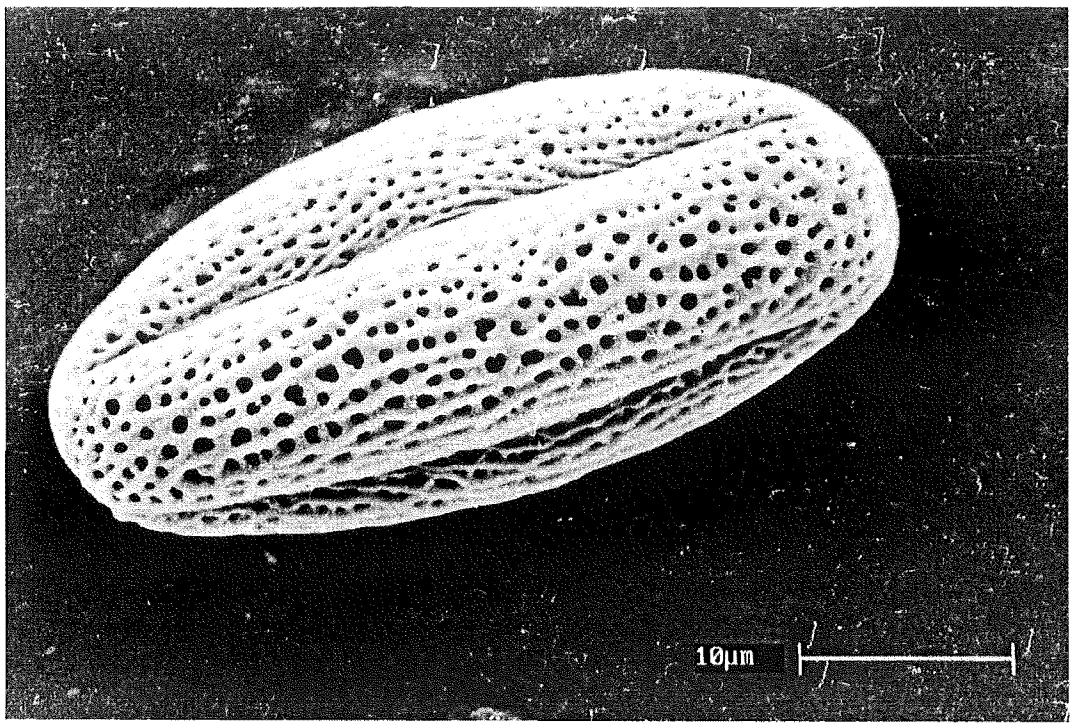


**Figure 3-16 A close-up SEM examination of a pollen grain from *in vitro* *G. triflora* flower**

An *in vitro* pollen grain of *G. triflora* was observed through SEM. This shows the horizontal feature of the pollen gain.


**Figure 3-17 Top view of pollen grain from *in vitro G. triflora* flower observed through SEM**

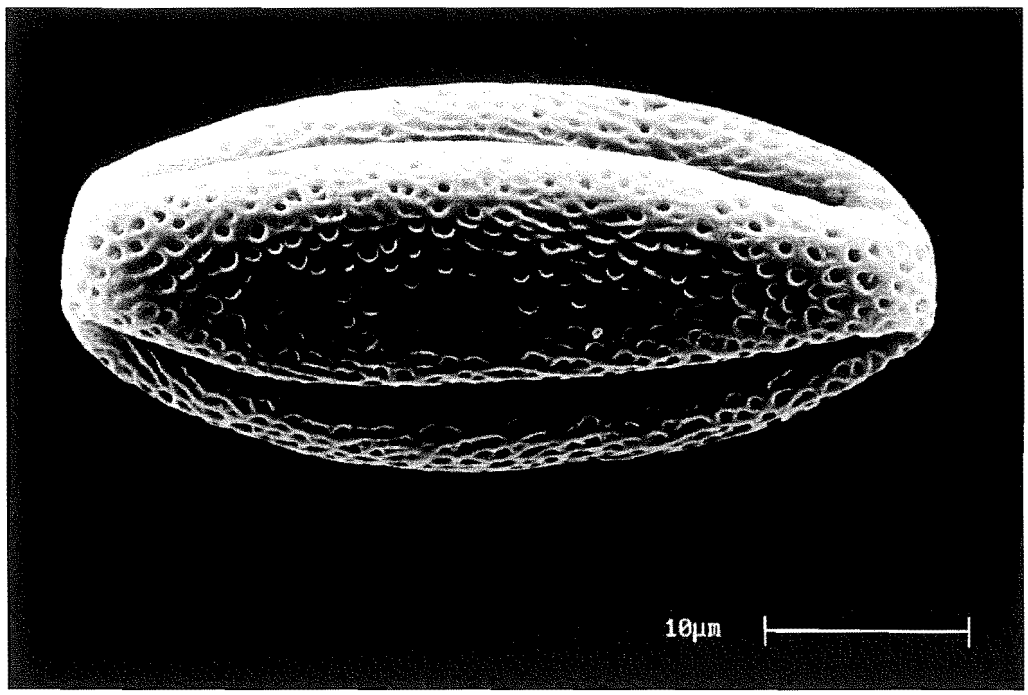


**Figure 3-18 A close-up SEM examination of a pollen grain from *in vivo* *G. triflora* flower**

An *in vivo* pollen grain of *G. triflora* was observed through SEM. This shows the horizontal feature of the pollen gain.

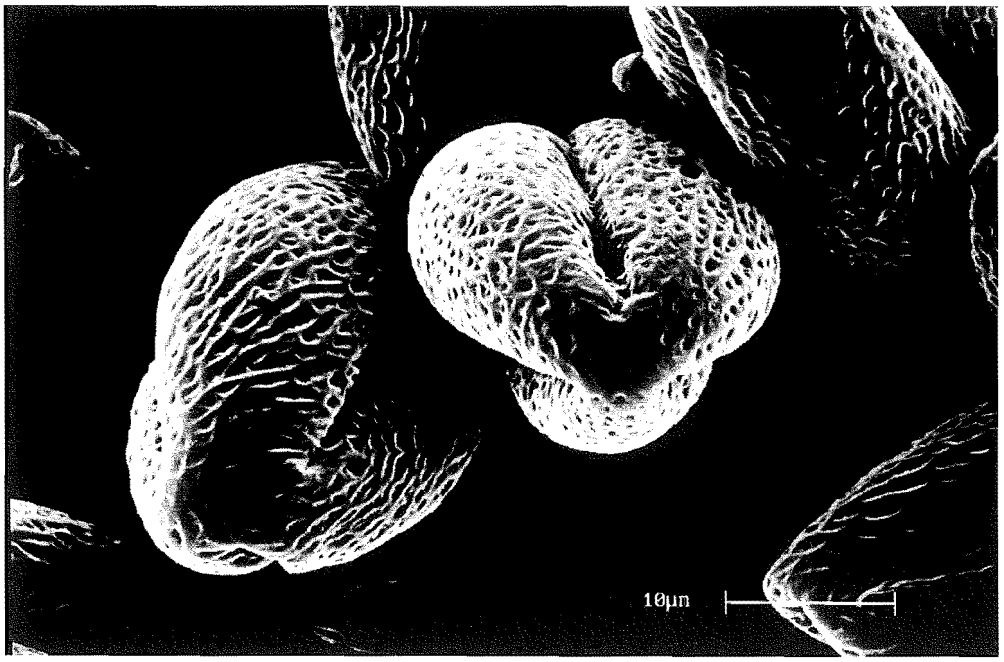


**Figure 3-19 Top view of pollen grains from *in vivo* flower of *G. triflora* observed through SEM**

### 3.1.7.3 Viability test of the pollen grains

Anthers were removed from both *in vitro* and *in vivo* flowers to obtain pollen for viability tests. Over 90% the pollen grains from both sources were viable as revealed by smearing and differential staining using Alexander's procedure (Alexander, 1969) (Figures 3-20 and 3-21; also see Table 3-1).

### 3.1.7.4 Germination test of the pollen grains

Pollen grains of *in vivo* flowers began to germinate in 1 hour and those of *in vitro* flowers after 5 hours. At the last observation (i.e. after 7 hours) from the start of the germination test, 10.7% and 33.7% of pollen from *in vitro* and *in vivo* flowers germinated, respectively (Figures 3-22 and 3-23; also see Table 3-1).

### 3.1.7.5 Pollination test

Gentian seeds are extremely small with 14,000 dry seeds per gram in the cultivar Akita Blue. Four weeks after hand pollination, no seed was formed at the end of *in vitro* flowering and few (3%) were obtained from those plants in the gentian farm (Table 3-1), which was 10-fold higher than the seed production without aided pollination (0.3%). Seed-like structures that were likely to be those unfertilized ovules of *G. triflora* from both *in vitro* and *in vivo* flowers were observed using scanning electron microscope (Figure 3-24). These structures were smaller than the few seeds obtained (Table 3-1) and light brown in colour.

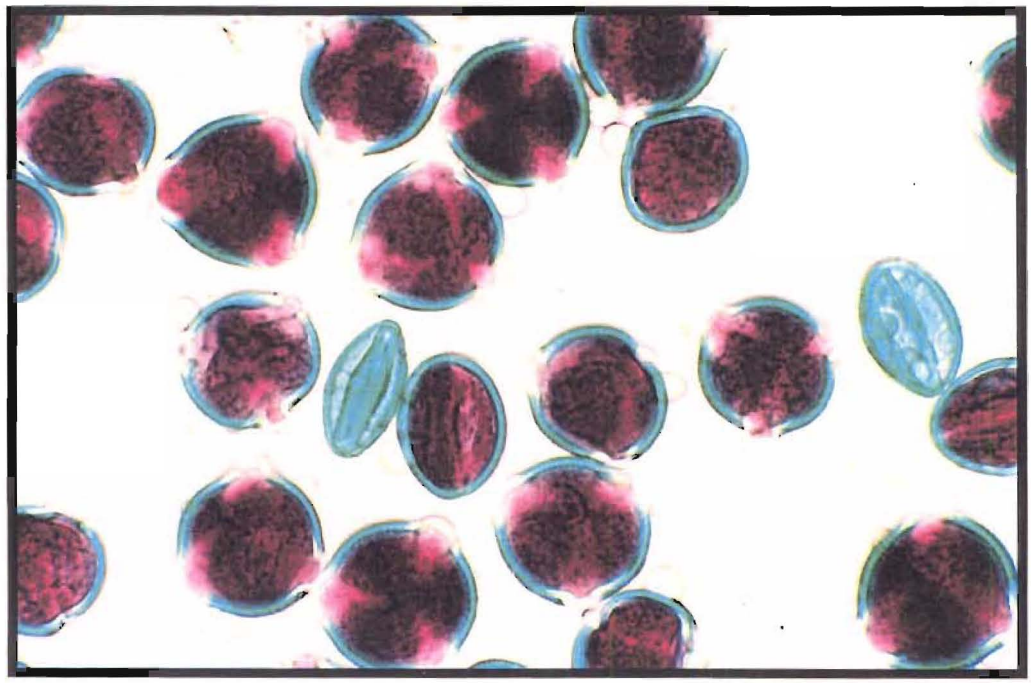

**Figure 3-20 Viability of *in vitro* pollen grains of *G. triflora***

Pollen viability was examined using the Alexander's procedure (Alexander, 1969). Those pollen grains stained (red) were viable.

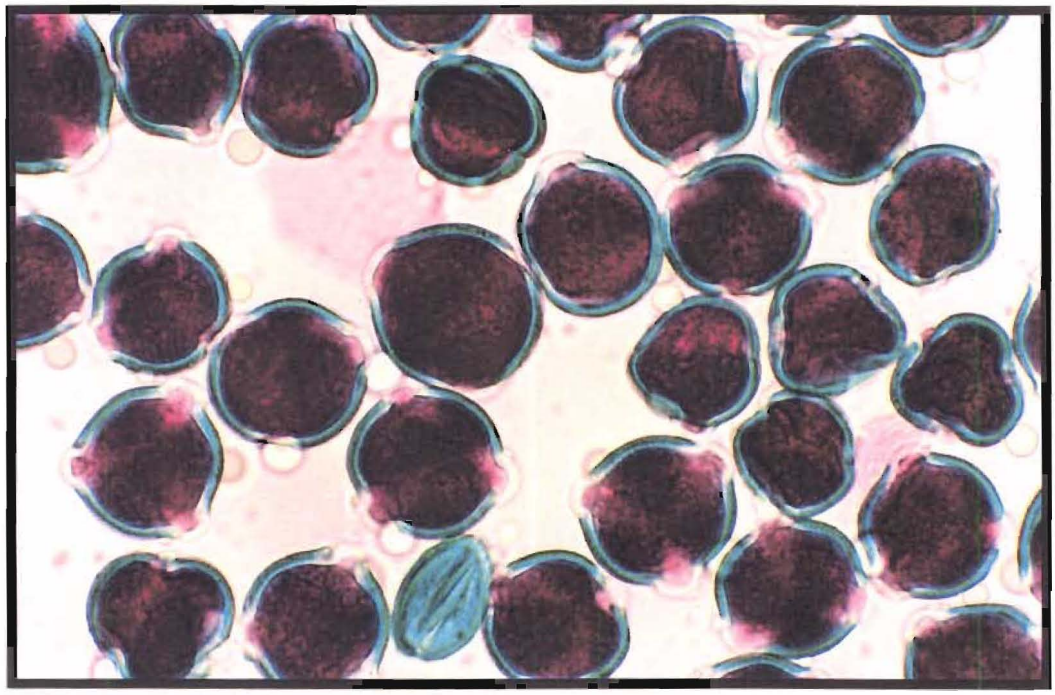

**Figure 3-21 Viability of *in vivo* pollen grains of *G. triflora***

Pollen viability was examined using the Alexander's procedure (Alexander, 1969). Those pollen grains stained (red) were viable.

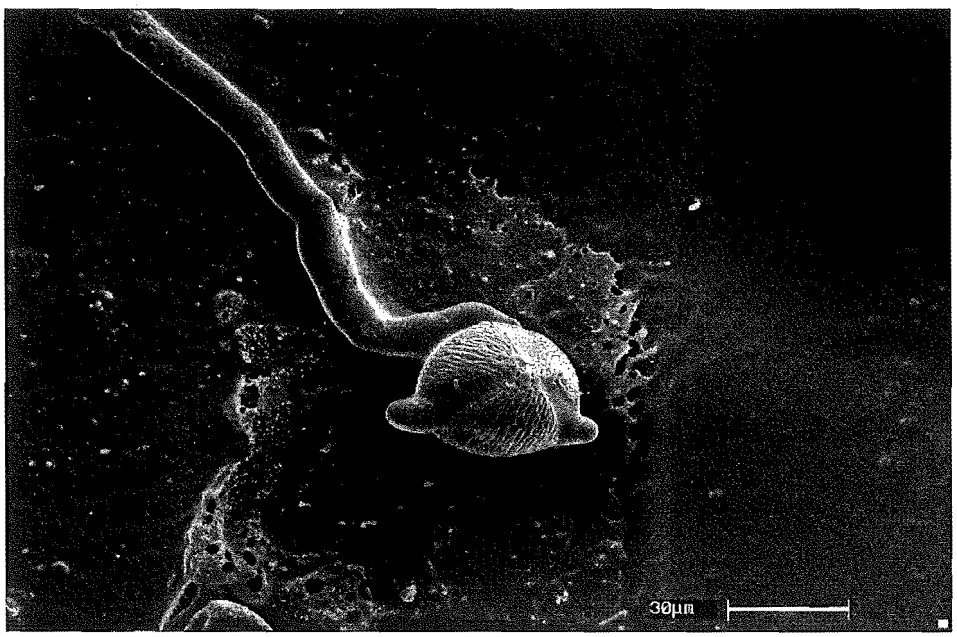


**Figure 3-22 Germination of a pollen grain from *in vitro* flower of *G. triflora***

Observations of *in vitro* pollen germination of *G. triflora* were made using SEM.

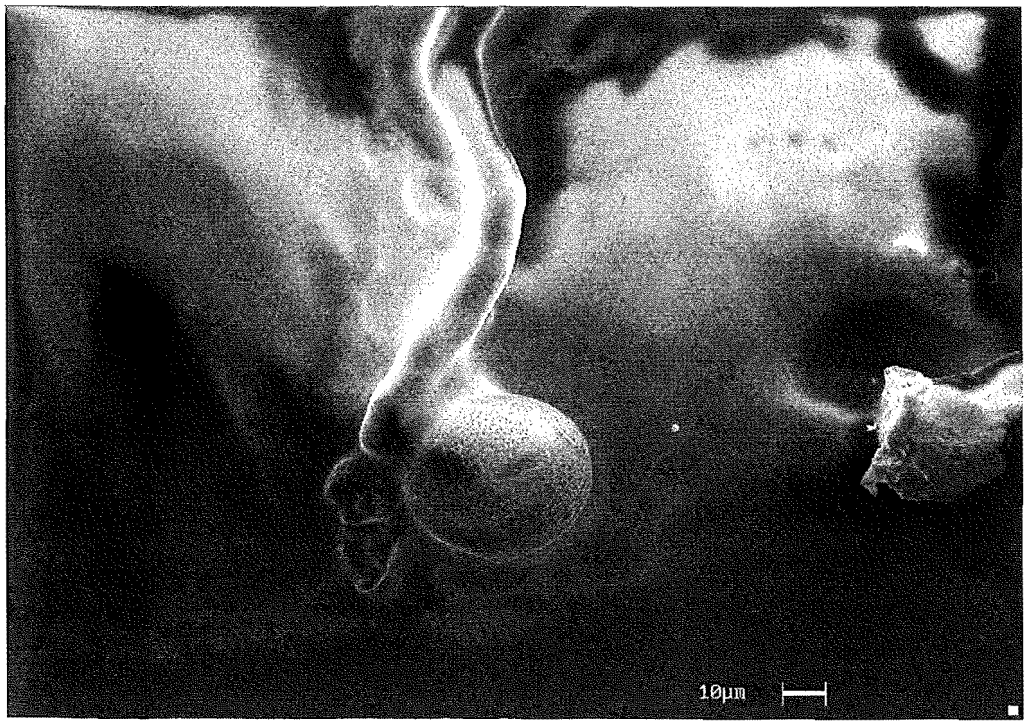


**Figure 3-23 Germination of a pollen grain of *in vivo* flower of *G. triflora***

Observations of *in vivo* pollen germination of *G. triflora* were made using SEM.

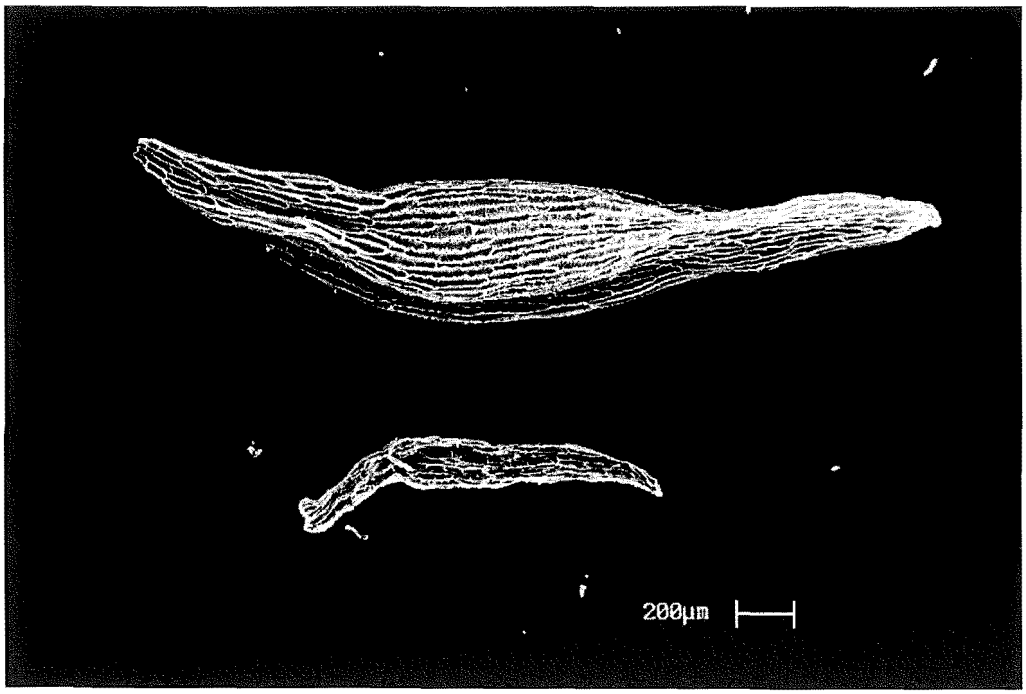


**Figure 3-24 Seed and seed-like structure of *G. triflora***

SEM observations were made to compare the seed (top panel) obtained from *in vivo* flowers with the much smaller seed-like structure (bottom panel) of *in vitro* flower of *G. triflora*. These structures were very fine and light brown in colour.

## 3.2. Post-harvest life of cut gentian flowers

### 3.2.1. *Effect of sucrose pulsing for 24 h*

The effect of pulsing cut *G. triflora* flower stems for 24 h with different concentrations of sucrose on the vase life of the flowers that were already open at the time of the pulsing treatments was investigated. The 3% (w/v) sucrose treatment was most effective in prolonging the vase life of the open flowers (Figure 3-25) among the treatments. Fresh weight of the petals was not affected by pulsing with low sucrose concentration at 1% (w/v) but it increased with the increased sucrose concentrations up to 5% (w/v) (Figure 3-26).

The white buds in the 3% sucrose (S + HQS) treatment became blue faster than the control and the blue buds opened two days earlier than those in the control (HQS alone) (Table 3-2). Moreover, the vase life of the open flowers in the S + HQS treatment was improved by 4 days compared with the control.

### 3.2.2. *Effect of STS pulsing for 24 h*

The effect of pulsing cut *G. triflora* flower stems for 24 h with different concentrations of STS on the vase life of the flowers that were already open at the time of the pulsing treatments was also investigated. The 0.5 mM STS treatment was most effective in prolonging the vase life of the open flowers (Figure 3-27) among the treatments. White buds in the cut stems pulsed with 0.5 mM STS (STS + HQS) became blue faster than the other two treatments (Table 3-2). A comparison of S + HQS with STS + HQS revealed that overall STS at the concentration of 0.5 mM was much more effective in improving all 3 developmental aspects studied as shown in Table 3-2. Fresh weight of the petals increased with the increased STS concentrations used for pulsing (Figure 3-28).

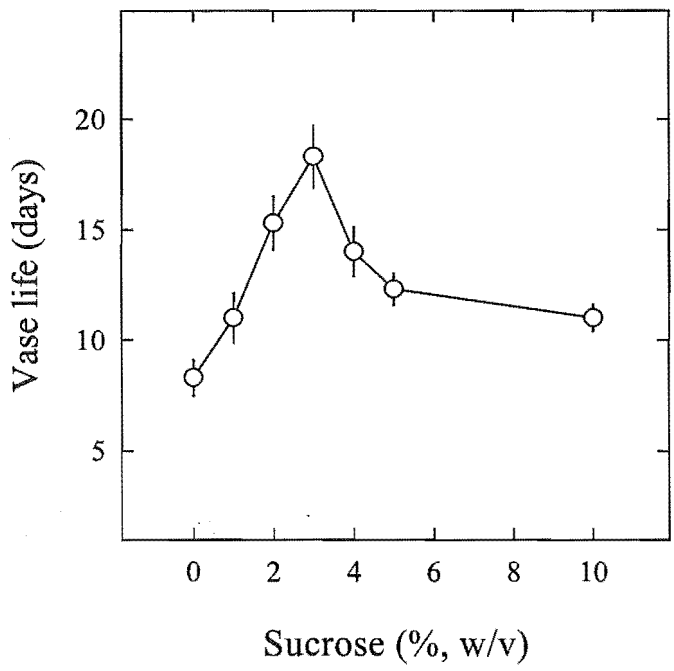


**Figure 3-25 Vase life of open flowers of cut gentian stems pulsed with sucrose**

After 24 hours of pulsing with different concentrations of sucrose, the stems were transferred to de-ionized water for vase life assessment in a growth room at 22°C, with 70% relative humidity, and at PPFD of 60 $\mu$mol · $m^{-2}$ · $s^{-1}$ irradiance using cool white fluorescent tubes for 16 hours daily. The mean number of days ± standard errors for the open flowers to start wilting (i.e. vase life) in a treatment was determined. The experiments were repeated twice, each with 5 replicates per treatment. Statistical analysis (ANOVA) showed a significant difference among the treatments ($p< 0.05$).

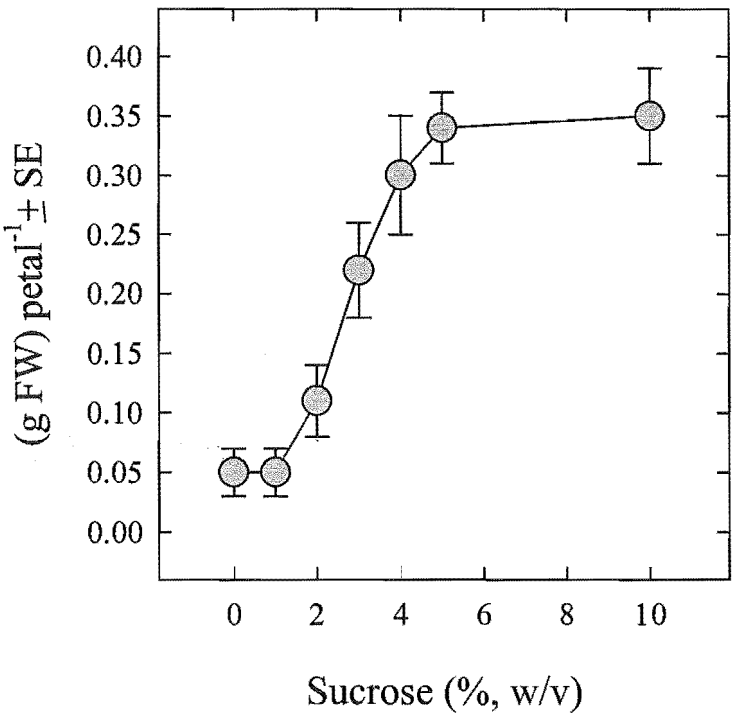


**Figure 3-26 Fresh weights of open *in vitro* gentian flowers after pulsing for 24 h with different concentrations of sucrose**

The flowers were pulsed with sucrose at different concentration from 1 to 10% (w/v) for 24 hours at room temperature. Petals from each treatment were freshly weighed individually. The experiments were repeated 3 times, each with 5 replicates per treatment.

**Table 3-2 Effect of sucrose and STS pulsing treatments on the development of white and blue buds, and vase life of open gentian flowers**

| Developmental stages | Treatments | Colour changes[+] | Buds open[++] | Start of petal wilting[+++] |
|---|---|---|---|---|
| White Buds | HQS[1] | 5 ± 0.2b | 5 ± 0.2b | 8 ± 0.9a |
| | S+HQS[2] | 4 ± 0.2b | 3 ± 0.2a | 12 ± 0.6b |
| | STS+HQS[3] | 1 ± 1.7a | 4 ± 0.0ab | 24 ± 9.8c |
| Blue Buds | HQS[1] | - | 4 ± 0.2b | 8 ± 0.8a |
| | S+HQS[2] | - | 3 ± 0.2b | 12 ± 0.9b |
| | STS+HQS[3] | - | 1 ± 0.6a | 24 ± 4.9c |
| Open Flowers | HQS[1] | - | - | 6 ± 0.5a |
| | S+HQS[2] | - | - | 14 ± 1.2b |
| | STS+HQS[3] | - | - | 16 ± 6.4b |

Values are mean days ± SE to display the developmental stages indicated. The experiments were repeated 3 times, each with 5 replicates per treatment. Data marked by the same letter in a column are not significantly different (Tukey's multiple comparison test, $p< 0.05$).

[1]. 8-hydroxyquinoline sulfate ($200mgl^{-1}$)

[2]. Sucrose at 87.6mM with 8-hydroxyquinoline sulfate ($200mgl^{-1}$)

[3]. STS at 0.5mM plus 8-hydroxyquinoline sulfate ($200mgl^{-1}$)

+. White buds to become blue buds

++. Blue buds to become open flowers

+++. Open flowers start to lose turgor

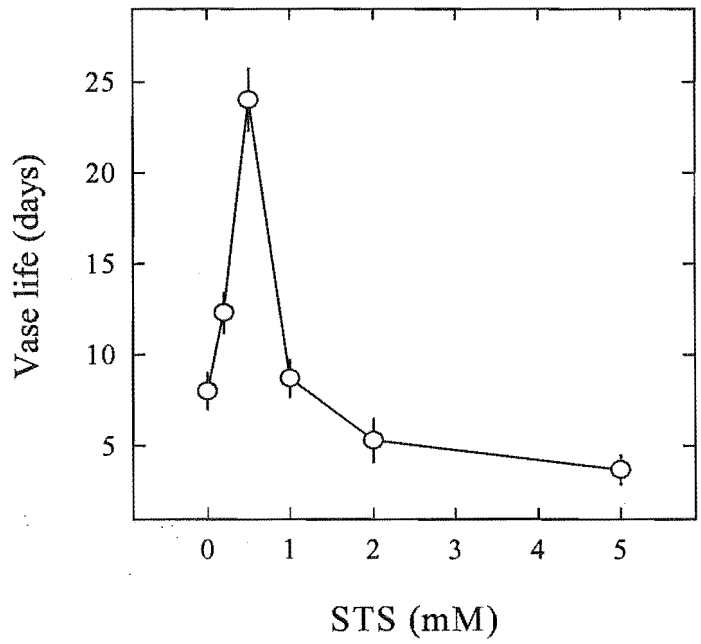


**Figure 3-27 Vase life of open flowers of cut gentian stems pulsed with STS**

After 24 hours of pulsing with different concentrations STS, the stems were transferred to de-ionized water for vase life assessment in a growth room at 22°C, with 70% relative humidity, and at PPFD of 60 $\mu$mol $m^{-2}$ $s^{-1}$ irradiance using cool white fluorescent tubes for 16 hours daily. The mean number of days ± standard errors for the open flowers to start wilting (i.e. vase life) in a treatment was determined. The experiments were repeated twice, each with 5 replicates per treatment. Statistical analysis (ANOVA) showed a significant difference among the treatments ($p< 0.05$).

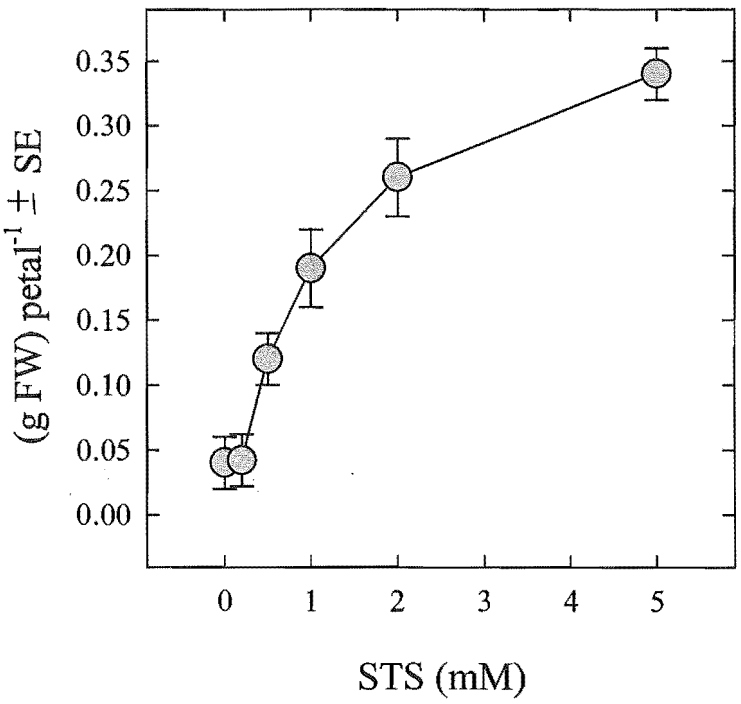


**Figure 3-28 Fresh weight of *in vitro* gentian flowers after pulsing for 24 h with different concentrations of STS**

The flowers that were open were pulsed with different concentrations of STS for 24 hours at room temperature. Petals from each treatment were weighed immediately after pulsing. The experiments were repeated 3 times, each with 5 replicates per treatment.

### 3.2.3. *Changes in fresh weight of gentian flower after various treatments*

Fresh weight of petals and reproductive parts of gentian flowers were increased obviously after pulsing with 3% sucrose or 0.5 mM STS at blue bud and open flower stages comparing with those untreated flowers and the controls (compare Table 3-3, Table 3-4 and Table 3-5). In the wilted flowers, the fresh weight of the petals remained unchanged but increased in reproductive parts after pulsing with 3% sucrose or 0.5 mM STS (Table 3-6). A notable phenomenon was that the fresh weight did not seem to change in the sepals in all cases tested regardless of either the stages of the flowers or the pulsing with 3% sucrose or 0.5 mM STS (Tables 3-3 to 3-6).

### 3.2.4. *Effect of osmotically equivalent carbohydrate solutions for pulsing*

The vase life of open gentian flowers was about 15 days following pulsing the cut stems with sucrose, fructose and glucose solutions that were osmotically equivalent to 3% sucrose (Figure 3-29). In contrast, the vase life of those in the control and the sorbitol pulsing treatments was much shorter.

### 3.2.5. *Changes in soluble sugar content following 24-h pulsing with carbohydrates and STS*

Soluble sugar concentrations in petals of open *G. triflora* flowers were affected by the pulsing treatments. During the 24-h pulsing treatment with fructose, glucose, sucrose and STS, the soluble sugar contents in the petals increased to a similarly higher level than that before pulsing (Table 3-7). In contrast, there was no increase in soluble sugar contents of the petals in the water control and the pulsing treatment with sorbitol.

Statistical analysis (ANOVA) established a highly significant difference in total soluble sugar content among the treatments ($p < 0.001$). The high level of soluble sugar contents in the fructose-, glucose- and sucrose-pulsed petals were maintained for up to day 8, while that in the STS-pulsed petals was maintained up to day 12. The low level of soluble sugar in control and sorbitol-pulsed petals appeared to diminish further immediately after pulsing treatments (Table 3-7).

### 3.2.6. *Changes in starch content following 24-h pulsing with carbohydrates and STS*

One day after the pulsing treatments with the carbohydrates or water the petals seem to have higher levels of starch than those treated with STS (Figure 3-30). After this, the general patterns of changes in starch level among the pulsing treatments with sorbitol, fructose, sucrose, water and STS are similar. In these treatments, starch level in the petals appeared to rise from day 2 before it began to drop at day 6 except in the STS treatment that displayed a drop from day 2. The petals pulsed with glucose initially appeared to have a high level of starch which was then lowered continuously from day 1 till the end of the experiment. From day 2 to day 4, the sorbitol-pulsed petals appeared to have more starch than that in the other treatments.

**Table 3-3 Fresh weight of untreated flower parts of *G. triflora* at different developmental stages**

| Flower parts | Stages | Fresh weight (g) |
|---|---|---|
| Petals | Blue bud | 0.12 ± 0.01c |
| | Open flower | 0.25 ± 0.02a |
| | Wilted flower | 0.16 ± 0.02b |
| Sepals | Blue bud | 0.04 ± 0.01a |
| | Open flower | 0.04 ± 0.01a |
| | Wilted flower | 0.04 ± 0.00a |
| Reproductive parts | Blue bud | 0.13 ± 0.03a |
| | Open flower | 0.16 ± 0.01a |
| | Wilted flower | 0.13 ± 0.02a |

Values are means of 3 replicates ± SE. Data marked by the same letter in a column are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-4 Fresh weight of flower parts of *G. triflora* at blue bud stage after 24 h pulsing by sucrose or STS**

| Flower parts | Treatments | Fresh weight (g) |
|---|---|---|
| Petals | Control | 0.10 ± 0.01b |
| | Sucrose (3%) | 0.23 ± 0.02a |
| | STS (0.5mM) | 0.23 ± 0.02a |
| Sepals | Control | 0.04 ± 0.01a |
| | Sucrose (3%) | 0.05 ± 0.01a |
| | STS (0.5mM) | 0.05 ± 0.00a |
| Reproductive parts | Control | 0.11 ± 0.01b |
| | Sucrose (3%) | 0.21 ± 0.03a |
| | STS (0.5mM) | 0.20 ± 0.02a |

Values are means of 3 replicates ± SE. Data marked by the same letter in a column are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-5 Fresh weight of flower parts of *G. triflora* at open stage after 24 h pulsing by sucrose or STS**

| Flower parts | Treatments | Fresh weight (g) |
|---|---|---|
| Petals | Control | 0.12 ± 0.02b |
| | Sucrose (3%) | 0.33 ± 0.02a |
| | STS (0.5mM) | 0.32 ± 0.02a |
| Sepals | Control | 0.04 ± 0.01b |
| | Sucrose (3%) | 0.06 ± 0.01a |
| | STS (0.5mM) | 0.06 ± 0.00a |
| Reproductive parts | Control | 0.13 ± 0.01b |
| | Sucrose (3%) | 0.24 ± 0.03a |
| | STS (0.5mM) | 0.22 ± 0.02a |

Values are means of 3 replicates ± SE. Data marked by the same letter in a column are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-6 Fresh weight of flower parts of *G. triflora* at the stage when flower wilted after 24 h pulsing by sucrose or STS**

| Flower parts | Treatments | Fresh weight (g) |
|---|---|---|
| Petals | Control | 0.11 ± 0.02a |
| | Sucrose (3%) | 0.12 ± 0.02a |
| | STS (0.5mM) | 0.13 ± 0.02a |
| Sepals | Control | 0.04 ± 0.00a |
| | Sucrose (3%) | 0.04 ± 0.01a |
| | STS (0.5mM) | 0.05 ± 0.00a |
| Reproductive parts | Control | 0.12 ± 0.01b |
| | Sucrose (3%) | 0.19 ± 0.03a |
| | STS (0.5mM) | 0.18 ± 0.02a |

Values are means of 3 replicates ± SE. Data marked by the same letter in a column are not significantly different ($p < 0.05$) according to the Tukey's test.

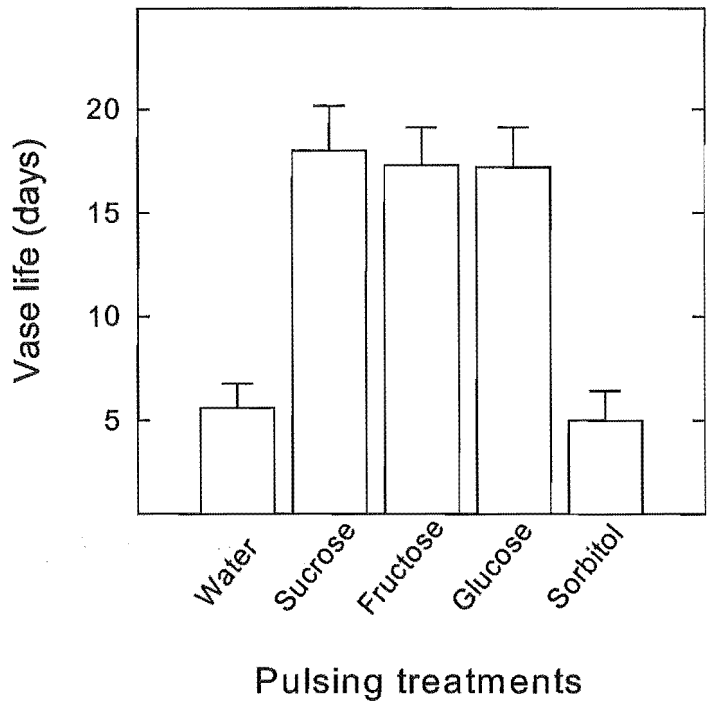


**Figure 3-29 Vase life of open flowers of cut gentian stems pulsed with different carbohydrate solutions containing HQS**

After 24 hours of pulsing with HQS alone (control) or various carbohydrates at the concentration that is osmotically equivalent to 3% sucrose, i.e. 87.6mM of glucose, fructose or sorbitol, the stems were transferred to de-ionized water for vase life assessment in a growth room at 22°C, with 70% relative humidity, and at PPFD of 60 $\mu$mol $m^{-2}$ $s^{-1}$ irradiance using cool white fluorescent tubes for 16 hours daily. The mean number of days ± standard errors for the open flowers to start wilting (i.e. end of vase life) in a treatment was determined. The experiments were repeated twice, each with 5 replicates per treatment. Statistical analysis (ANOVA) showed a highly significant difference among the treatments ($p< 0.01$).

**Table 3-7 Effect of 24-h pulsing with carbohydrates (87.6mM) and STS (0.5mM) on total soluble sugar content (mg/gFW) in petals of gentian flowers**

| Days | STS | Sucrose | Fructose | Glucose | Control | Sorbitol |
|---|---|---|---|---|---|---|
| 01* | 2.5 ± 0.10a | 2.4 ± 0.12a | 2.5 ± 0.06a | 2.4 ± 0.12a | 2.6 ± 0.21a | 2.5 ± 0.06a |
| 02** | 3.9 ± 0.26a | 3.6 ± 0.10ab | 3.6 ± 0.27ab | 3.5 ± 0.06ab | 2.3 ± 0.35b | 2.2 ± 0.32b |
| 2 | 3.9 ± 0.20a | 3.6 ± 0.10ab | 3.6 ± 0.12ab | 3.6 ± 0.06ab | 1.6 ± 0.20b | 1.5 ± 0.12b |
| 4 | 3.8 ± 0.10a | 3.6 ± 0.06a | 3.8 ± 0.25a | 3.6 ± 0.10a | 1.6 ± 0.10b | 1.2 ± 0.16c |
| 6 | 3.8 ± 0.17a | 3.6 ± 0.17a | 3.6 ± 0.06a | 3.8 ± 0.25a | 1.0 ± 0.12b | 1.2 ± 0.17b |
| 8 | 3.8 ± 0.10a | 3.6 ± 0.20a | 3.6 ± 0.23a | 3.7 ± 0.10a | 1.0 ± 0.06b | n.d. |
| 10 | 3.8 ± 0.27a | 2.5 ± 0.06b | 2.6 ± 0.10b | 2.7 ± 0.12b | n.d. | n.d. |
| 12 | 3.9 ± 0.27a | 2.4 ± 0.17b | 2.5 ± 0.09b | 2.2 ± 0.17b | n.d. | n.d. |
| 14 | 2.8 ± 0.36a | 1.0 ± 0.15b | 1.2 ± 0.10b | 1.0 ± 0.17b | n.d. | n.d. |

Values are means ± SE of 3 experiments, each with 5 replicates per treatment. Data marked by the same letter in a row are not significantly different ($p < 0.05$) by Tukey's multiple comparison test.

* The day prior to pulsing

** The day immediately after 24-h pulsing

n.d. Not determined as flowers wilted

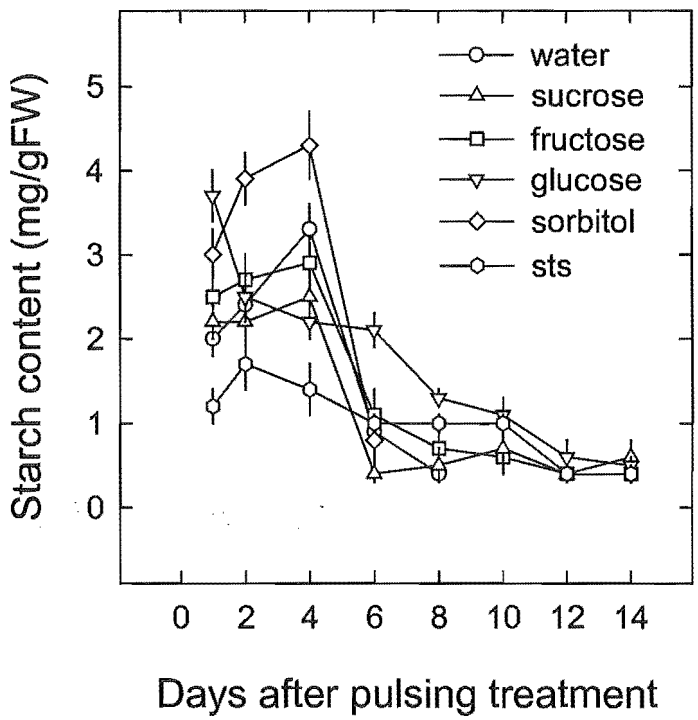


**Figure 3-30 Effect of 24-h pulsing treatments with carbohydrates or STS on starch content in the petals of gentian flowers**

The flowers were pulsed with different carbohydrates at the concentration of 87.6 mM or STS at 0.5mM for 24 hours at room temperature. Flowers were transferred to de-ionized water and kept in a growth room with 70% relative humidity, PPFD of 60 $\mu$mol · $m^{-2}$ · $s^{-1}$ irradiance using cool white fluorescence tubes at 22°C for 16 hours daily. Changes in average starch content ± SE in the petals after various pulsing treatments were determined on the days indicated. The experiments were repeated 3 times, each with 5 replicates per treatment.

### 3.2.7. *Changes in ethylene production after the pulsing treatments*

Immediately following the pulsing treatments, ethylene production from gentian petals was significantly different among the treatments (Table 3-9). The petals from flower stems pulsed with water evolved substantially more ethylene than those pulsed with sucrose or STS. Furthermore, STS pulsing led to the lowest level of ethylene production. By day 4 after the pulsing treatments, the petals of the water control evolved substantially less ethylene than before, which was still at a level higher than the other two pulsing treatments. At day 6, there was a dramatic rise in ethylene production by petals of the control before dropping to a much lower level at day 8. In the sucrose and STS treatments, the same pattern of changes in ethylene production between days 4-8 was also evident except that the rise in ethylene production at day 6 was significantly less than in the control. After day 6, ethylene production in all 3 treatments was very low or below detectable level with GC.

### 3.2.8. *Autocatalytic ethylene production*

Ethylene production by petals was measured following removal from the ethylene atmosphere. Treatment of flowers with 20 $\mu l\ l^{-1}$ ethylene for 24 h resulted in shortened petal longevity and induction of autocatalytic ethylene production (Table 3-10).

### 3.2.9. *$^{14}C$ Sucrose studies*

The use of $^{14}C$ sucrose in the pulsing solution indicated that after 24 h of pulsing there were 3 times more radioactivity in the 80% ethanol-soluble fraction (presumably soluble sugar) than in the perchloric acid extract of the ethanol-extract of insoluble fraction (i.e. starch) (Table 3-12).

**Table 3-9 The ethylene production rates (nl/gfw/h) of gentian flowers after pulsing for 24 h with water, sucrose (87.6mM) or STS (0.5mM)**

| Days* | 0 | 2 | 4 | 6 | 8 | 10 | 12 |
|---|---|---|---|---|---|---|---|
| Water | 28.0 ± 2.0a | - | 4.0 ± 0.8a | 31.0 ± 2.0a | 2.0 ± 0.5a | - | - |
| Sucrose | 5.1 ± 1.0b | - | 0.8 ± 0.5b | 13.1 ± 1.2b | 0.2 ± 0.1b | - | - |
| STS | 3.9 ± 0.7b | - | 0.7 ± 0.2b | 9.0 ± 2.7b | 0.5 ± 0.3b | - | - |

Values are means ± SE of 2 experiments, each with 5 replicates per treatment. Data marked by the same letter in a column are not significantly different ($p < 0.05$) by Tukey's multiple comparison test.

* Time after the pulsing treatments

- Below detectable level with GC

**Table 3-10 Ethylene-induced gentian petal senescence and autocatalytic ethylene production**

| Treatment | Petal Longevity (d) | Ethylene Production ( $nl\ gfw^{-1}\ h^{-1}$) |
|---|---|---|
| **Control** | 6.0 ± 0.2b | 1.8 ± 0.3c |
| **Sucrose + $C_2H_4$** | 13.2 ± 1.1a | 3.4 ± 0.4b |
| **STS + $C_2H_4$** | 14.6 ± 1.0a | 2.8 ± 0.2bc |
| **$H_2O$ + $C_2H_4$** | 3.4 ± 0.2c | 11.8 ± 0.8a |

Values are means ± SE of 2 experiments, each with 5 replicates per treatment. Data marked by the same letter in a column are not significantly different ($p < 0.05$) by Tukey's multiple comparison test.

**Table 3-12 Measurement of $^{14}C$ radioactivity in different 80% (v/v) ethanol fractions of petal extracts of gentian flowers**

| Fractions of the petal extracts | $^{14}C$ radioactivity (kBq/gFW) |
|---|---|
| ethanol – soluble fraction (soluble sugar) | 128.94 ± 31.4 |
| ethanol – insoluble fraction (starch) | 43.88 ± 7.0 |

Values are means ± SE of 2 experiments, each with 4 replicates per treatment. Statistical analysis (Student's t-test) established a highly significant difference ($p < 0.05$) in the amount of radioactivity between the 2 fractions.

## 3.3. Further Biochemical Studies

### 3.3.1. *Amylase activity determination without pretreatment at 70℃*

Extracts prepared from petal, sepal and reproductive parts of gentian flowers at the 3 developmental stages (Figure 3-32) as described in section 2.4.4. were assayed for amylase activity. At both the blue bud and open flower stages (Figures 3-32 and 3-33), petal had clearly higher amylase activity than sepal and reproductive parts, while the amylase activity was very similar in both sepal and reproductive parts. In contrast, when the flower wilted, the amylase activity appeared obviously lower in petal comparing to the other two parts (Figure 3-34). In general, the enzyme activity in the extracts of the different flower parts was linear over an incubation period at 37°C for 60 min. In addition, the different flower parts appear to have optimal amylase activity at pH 6 (Figure 3-35).


**Figure 3-31 Appearance of *G. triflora* flower at 3 developmental stages of interests in this study**

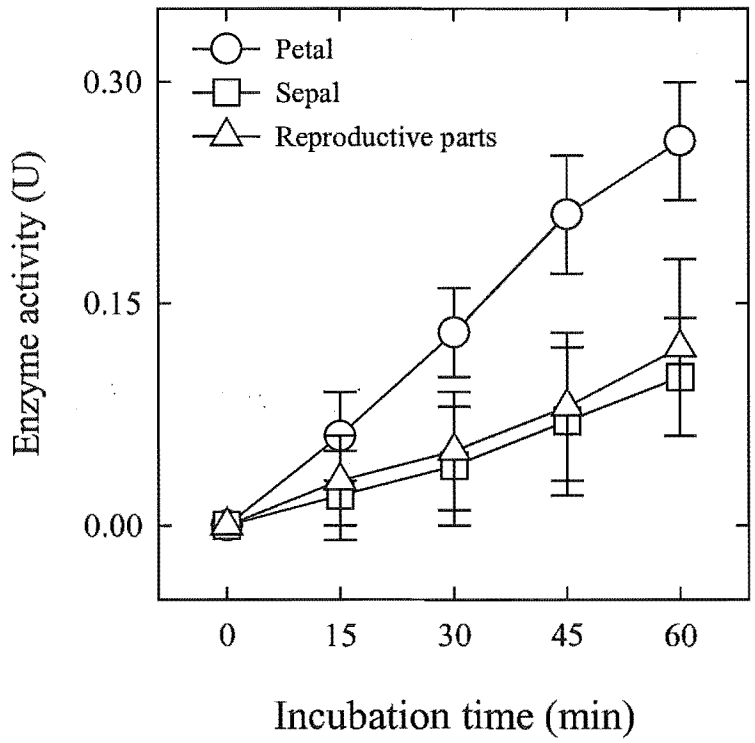


**Figure 3-32 Amylase activity measured at blue bud stage of *G. triflora***

50 µl of crude extracts prepared from petal, sepal and reproductive parts were incubated at 37°C for the time indicated for amylase activity determination. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Each data point is the mean (± SE) of 3 replicate enzyme assay reactions.

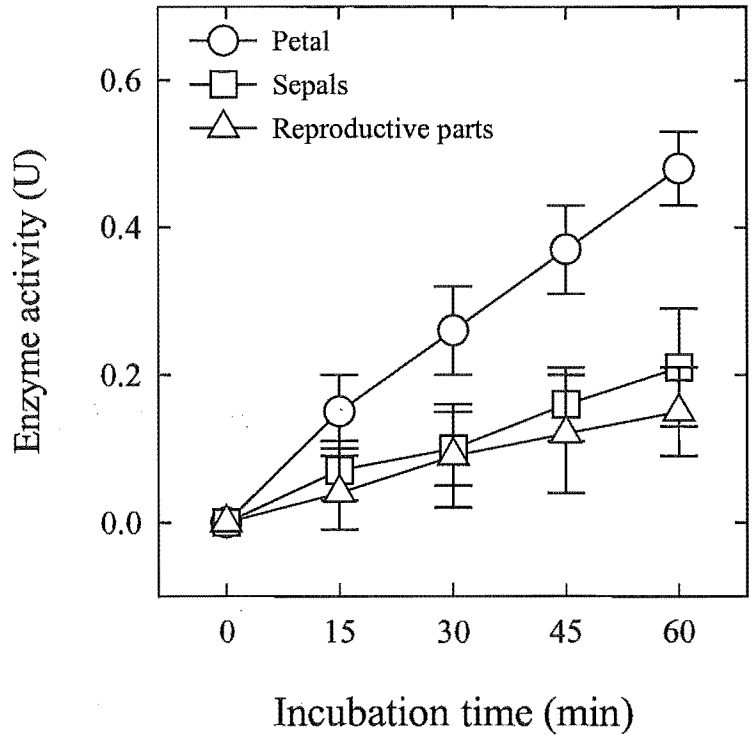


**Figure 3-33 Amylase activity measured at open flower stage of *G. triflora***

50 µl of crude extracts prepared from petal, sepal and reproductive parts were incubated at 37°C for the time indicated for amylase activity determination. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Each data point is the mean (± SE) of 3 replicate enzyme assay reactions.

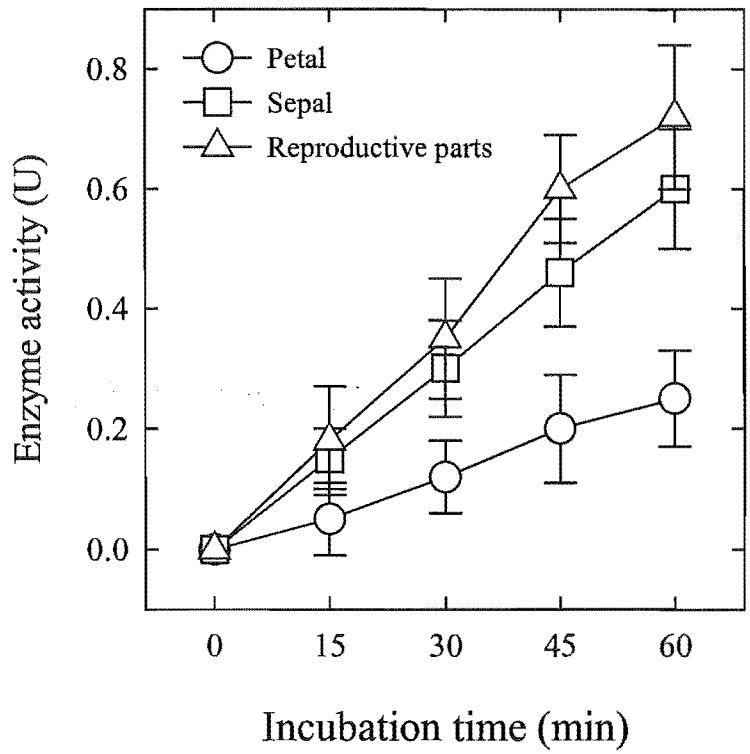


**Figure 3-34 Amylase activity measured at wilted flower stage of *G. triflora***

50 µl of crude extracts prepared from petal, sepal and reproductive parts were incubated at 37°C for the time indicated for amylase activity determination. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Each data point is the mean (± SE) of 3 replicate enzyme assay reactions.

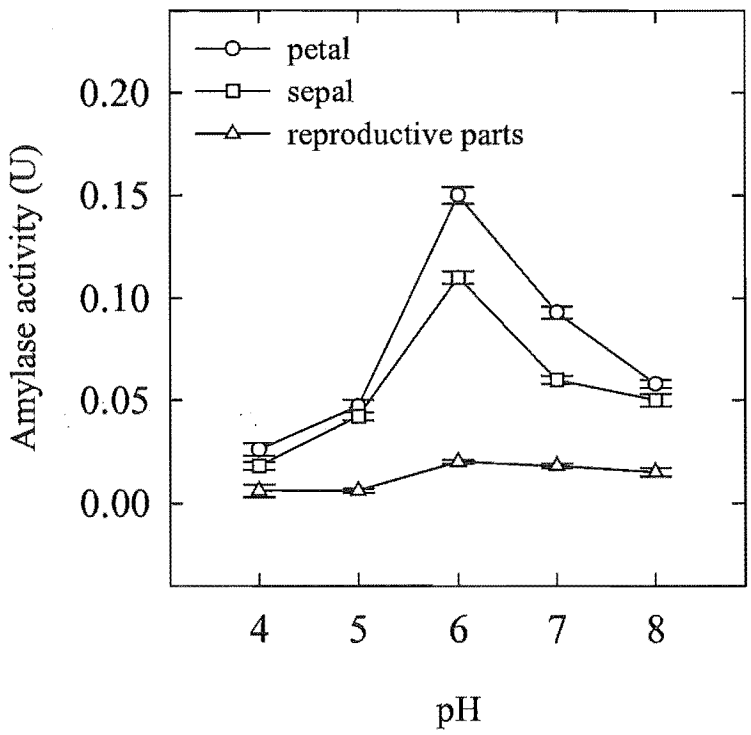


**Figure 3-35 Amylase activity in the extracts of different open *G. triflora* flower parts tested at different pH**

*In vitro G. triflora* open flower was collected and amylase activity was measured at different pH (4 –8) from different parts of the flower. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Each data value is the mean (± SE) of 3 replicate enzyme assay reactions.

### 3.3.2. *Determination of amylase activity in flower parts in response to sucrose or STS pulsing*

Immediately following pulsing for 24 h with water, 3% (w/v) sucrose or 0.5 mM STS, extracts of the petal, sepal and reproductive parts of gentian flower stems were assayed for amylase activity. The results showed that the amylase activity was generally higher in petal than in the other two flower parts. In each floral part, α-amylase activity was significantly higher in both the sucrose and STS treatments than in the water control. There was no significant difference in α-amylase activity between sucrose- and STS-pulsed flowers (Figure 3-36). After 24 hour with the 3 different pulsing treatments, amylase activity was the highest at day 4 before it began to decrease the day after in the 3 parts of gentian flower (Figures 3-37 to 3-39) except the reproductive parts in the water control where α-amylase activity gradually increased.

### 3.3.3. *Determination of hydrolysis products by paper chromatography*

Paper chromatographic analysis of the products generated by starch hydrolysis revealed glucose and discernible level of higher molecular weight substances (Figure 3-40). This suggests the presence of α-amylase activity in the different parts of *G.triflora* flower.

According to Briggs (1967), in the presence of excess $Ca^{2+}$, α-amylase activity is heat stable at 70°C for 5 min whereas that of β-amylase is denatured under these conditions. This was confirmed in this study (Figure 3-41). In the absence of $Ca^{2+}$, amylase activity in the extracts of different gentian flower parts was lower compared to heating at 70°C for 5 min in the presence of $Ca^{2+}$ (compare Figures 3-41 and 3-42). Quantitative enzyme activity data were paralleled with these results (Figures 3-43 to 3-45).

Further investigations indicated that the incubation of the extracts of different flower parts in the presence of 40 mM EDTA or at low pH (i.e. 3.0) for 15 min at 37°C had inhibited starch hydrolysis. In particular, glucose was not observed after both treatments (Figures 3-46 and 3-47).

### 3.3.4. *Time course of α-amylase activity development*

An aliquot of each the extracts prepared from petal, sepal and reproductive parts was heated at 70°C for 5 min to inactivate β-amylase (if any). After 15 min of incubation at 37°C amylase activity in both enzyme assay reactions was assayed by the modified method of Berfeld (1955) using 0.2% (w/v) soluble potato starch solution in 0.2 M acetate buffer (pH 5.2) (Appendix A2) as substrate. With this approach, it is confirmed that the amylase present in the extracts belongs to the α-type. The data showed that α-amylase activity was very low at the blue bud stage in the three floral parts of *G. triflora* (Figure 3-48). It had increased in all 3 parts when flower opened and appeared to increase further in the sepal and the reproductive parts till the flower became wilted. By contrast, α-amylase activity declined to a lower level in the petal when flower wilted. At the open flower stage, α-amylase activity was the highest in petals.

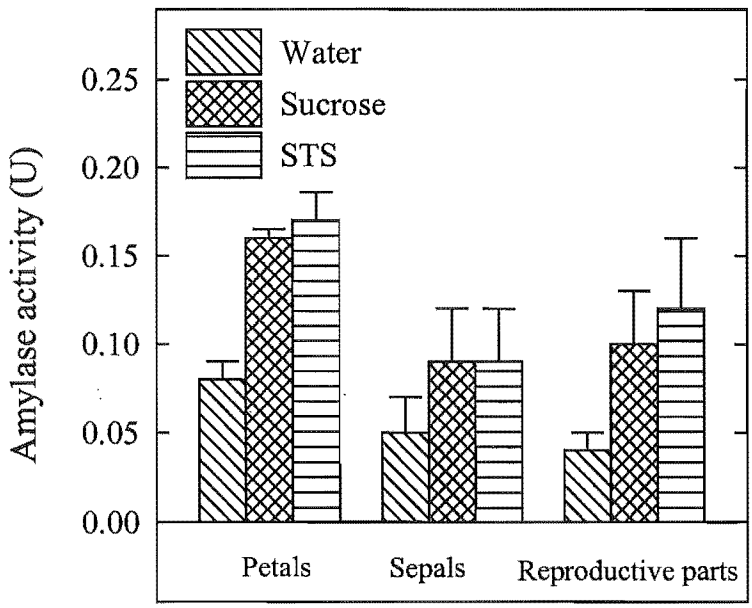


**Figure 3-36 Amylase activity in different flower parts in response to sucrose or STS pulsing**

Amylase activity in extracts of different parts from open gentian flowers was measured after 24 hour pulsing of the flower stems with water (control), 3% sucrose or 0.5 mM STS. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

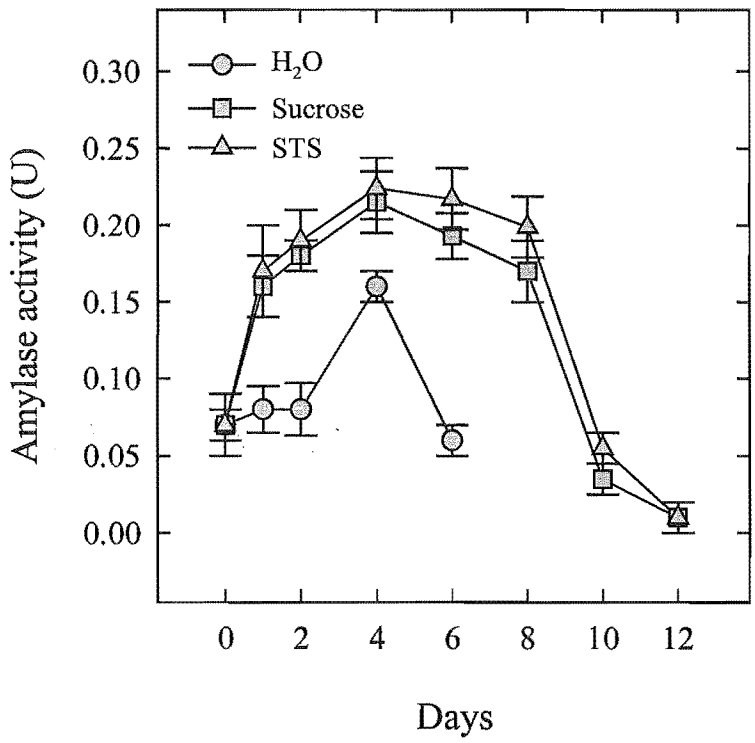


**Figure 3-37 Changes in α-amylase activity in the petal of *G. triflora* flower in response to different pulsing treatments**

Amylase activity in extracts of petal from *in vitro* open gentian flowers was measured before (day 0) and after 24 hour pulsing (day 1-12) of the flower stems with water (control), 3% sucrose or 0.5 mM STS. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

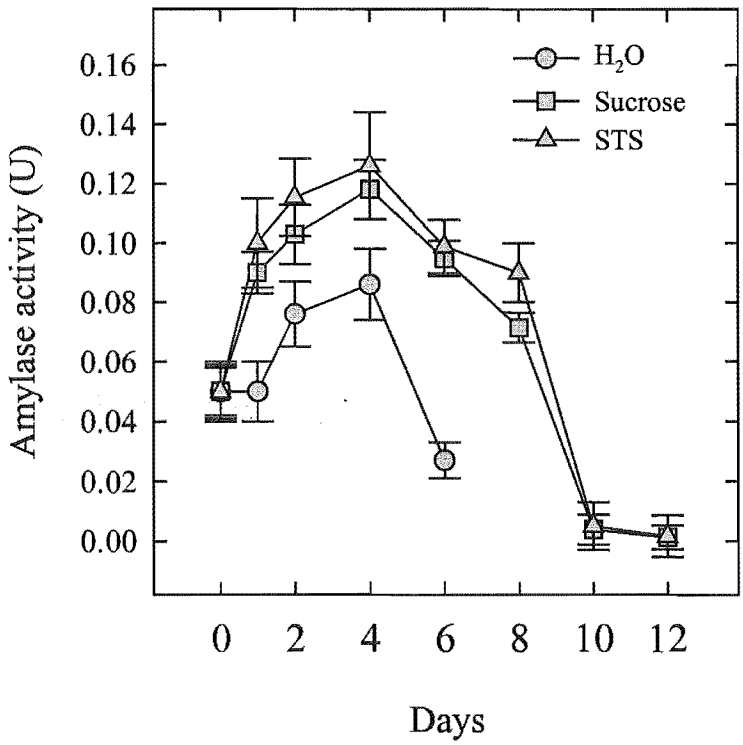


**Figure 3-38 Changes in α-amylase activity in the sepal of *G. triflora* flower in response to different pulsing treatments**

Amylase activity in extracts of sepal from *in vitro* open gentian flowers was measured before (day 0) and after 24 hour pulsing (day 1-12) of the flower stems with water (control), 3% sucrose or 0.5 mM STS. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

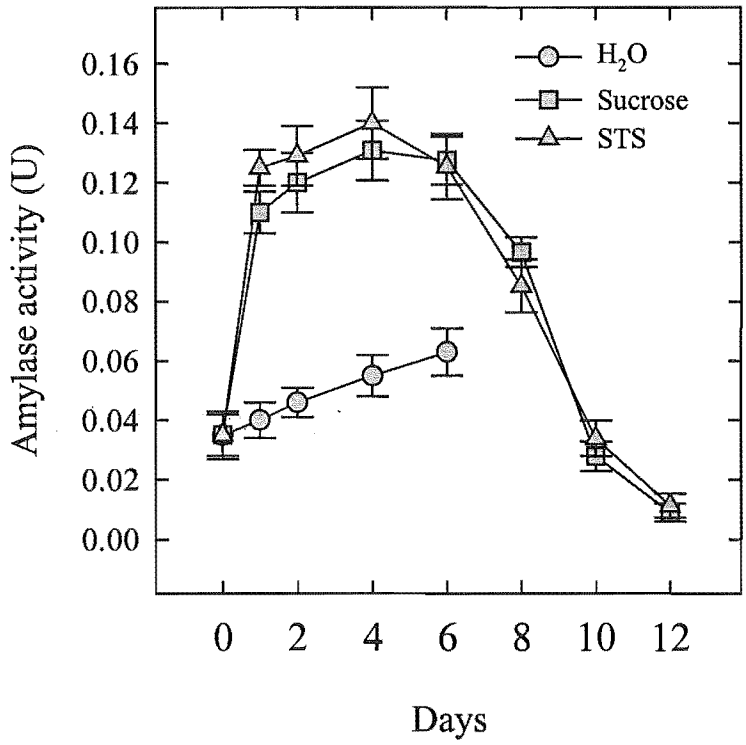


**Figure 3-39 Changes in α-amylase activity in the reproductive parts of *G. triflora* flower in response to different pulsing treatments**

Amylase activity in extracts of reproductive parts from *in vitro* open gentian flowers was measured before (day 0) and after 24 hour pulsing (day 1-12) of the flower stems with water (control), 3% sucrose or 0.5 mM STS. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

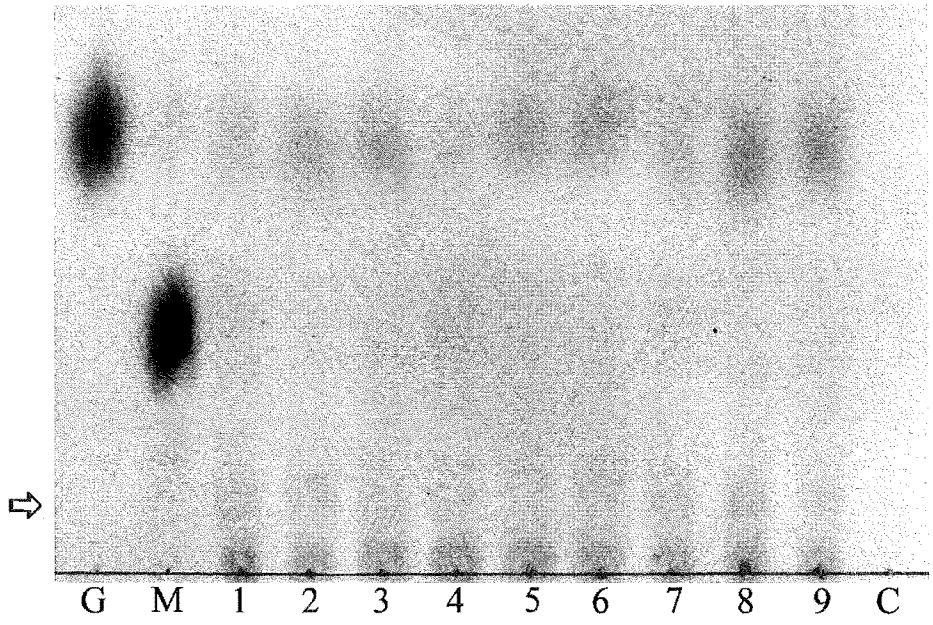


**Figure 3-40 Activity pattern of the amylase activity in the extracts of *G. triflora* flower parts on starch**

Extracts from different flower parts (lane # 1 to lane # 9) were incubated with 0.2% soluble potato starch solution at 37°C for 15 min. 20 µl samples were taken and spotted on Whatman No 1 paper. Sugars were resolved in ethyl acetate-pyridine-$H_2O$ (12:4:3). G and M: 5 µl of 1% glucose and 5 µl of 1% maltose standards, respectively; 1, 2 and 3: petals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 4, 5 and 6: sepals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 7, 8 and 9: reproductive parts from the flower pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; C: 20 µl control without CE in the reaction. The area pointed by ⇨ indicates the presence of high molecular weight substances.

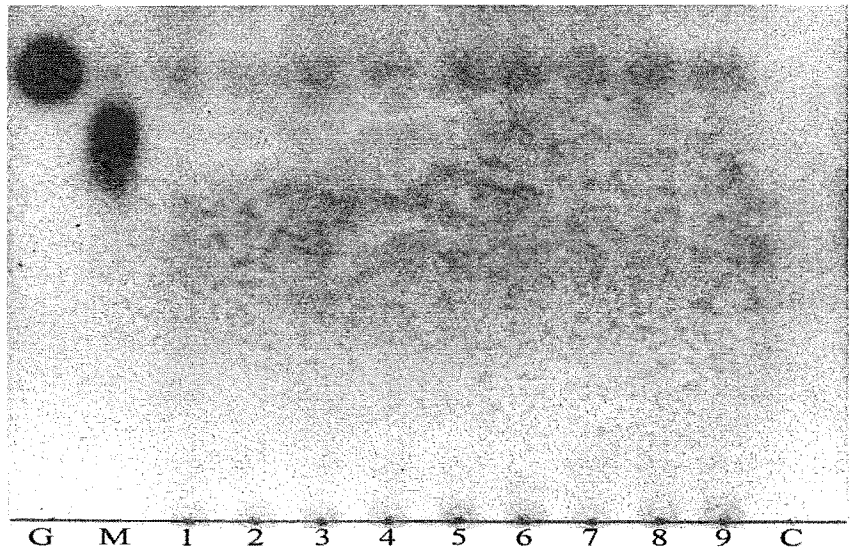


**Figure 3-41 Amylase activity in extracts of different gentian flower parts following heat treatment in the presence of $Ca^{2+}$**

Extracts from different flower parts (lane #1 to lane # 9) were incubated with 0.2% soluble potato starch solution in the presence of 10 mM $Ca^{2+}$ first at 70°C for 5 min and then at 37°C for 15 min. 20 µl samples were taken and spotted on Whatman No 1 paper. Sugars were resolved in ethyl acetate-pyridine-$H_2O$ (12:4:3). G and M: 5 µl of 1% glucose and 5 µl of 1% maltose standards, respectively; 1, 2 and 3: petals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 4, 5 and 6: sepals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 7, 8 and 9: reproductive parts from the flower pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; C: 20 µl control without CE in the reaction.

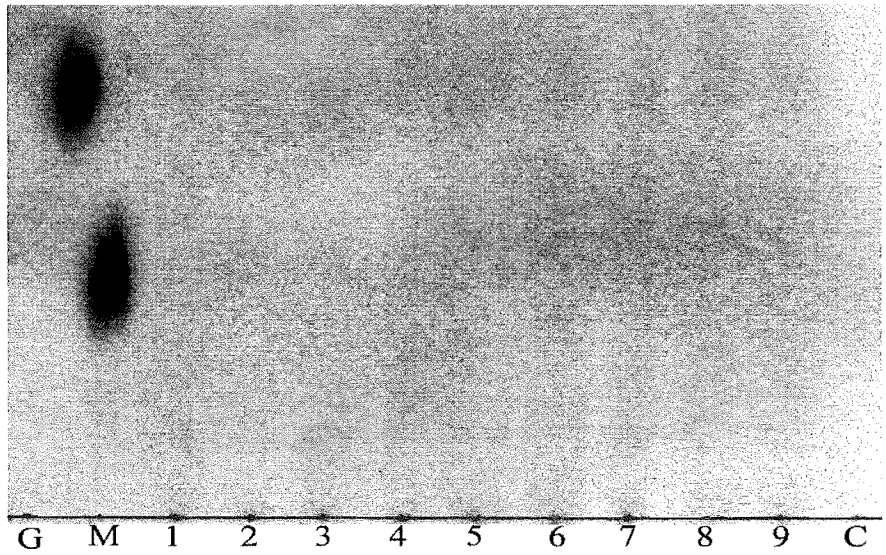


**Figure 3-42 Effect of heat treatment in the absence of $Ca^{2+}$ on amylase activity in extracts of different gentian flower parts**

Extracts from different flower parts (lane #1 to lane # 9) were incubated with 0.2% soluble potato starch solution in the absence of 10 mM $Ca^{2+}$ first at 70°C for 5 min and then at 37°C for 15 min. 20 µl samples were taken and spotted on Whatman No 1 paper. Sugars were resolved in ethyl acetate-pyridine-$H_2O$ (12:4:3). G and M: 5 µl of 1% glucose and 5 µl of 1% maltose standards, respectively; 1, 2 and 3: petals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 4, 5 and 6: sepals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 7, 8 and 9: reproductive parts from the flower pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; C: 20 µl control without CE in the reaction.

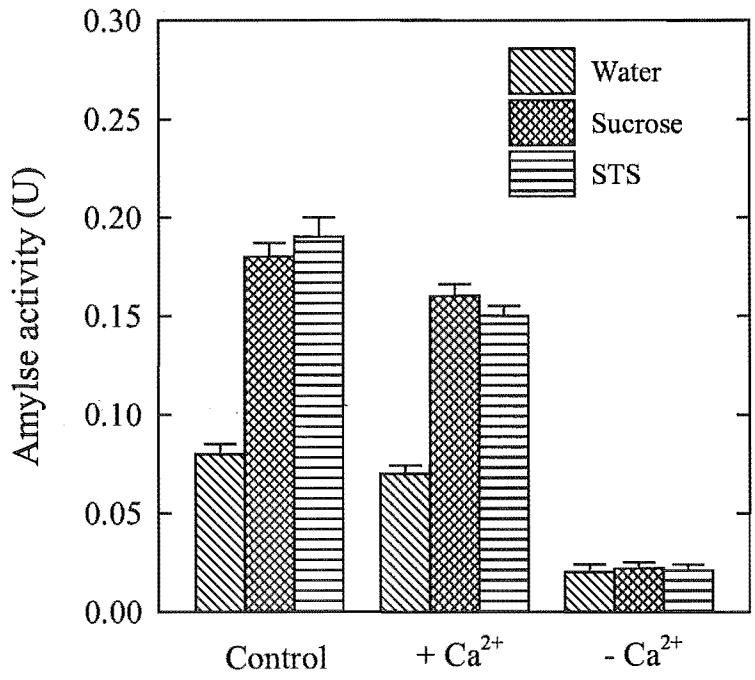


**Figure 3-43 Effect of heat treatment in the presence or absence of $Ca^{2+}$ on amylase activity in extracts of the petal of open gentian flower**

Extracts of the petal of open gentian flower from the stems pulsed for 24 h with water, 3% (w/v) sucrose or 0.5 mM STS were incubated with 0.2% soluble potato starch solution in the presence or absence of 10 mM $Ca^{2+}$ first at 70°C for 5 min and then at 37°C for 15 min. After this, amylase activity was measured immediately. The control assay reactions were carried out without heat treatment at 70°C for 5 min prior to incubation at 37°C for 15 min. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

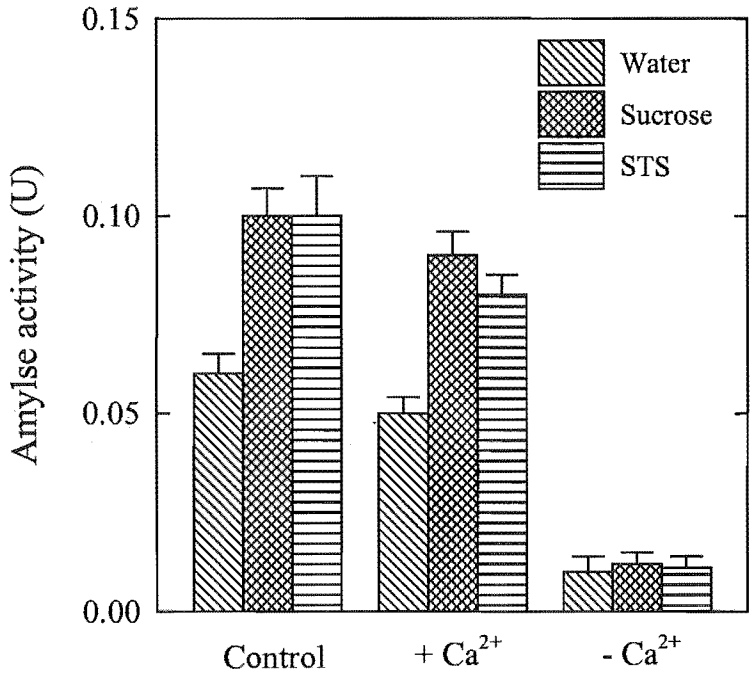


**Figure 3-44 Effect of heat treatment in the presence or absence of $Ca^{2+}$ on amylase activity in extracts of the sepal of open gentian flower**

Extracts of the sepal of open gentian flower from the stems pulsed for 24 h with water, 3% (w/v) sucrose or 0.5 mM STS were incubated with 0.2% soluble potato starch solution in the presence or absence of 10 mM $Ca^{2+}$ first at 70°C for 5 min and then at 37°C for 15 min. After this, amylase activity was measured immediately. The control assay reactions were carried out without heat treatment at 70°C for 5 min prior to incubation at 37°C for 15 min. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

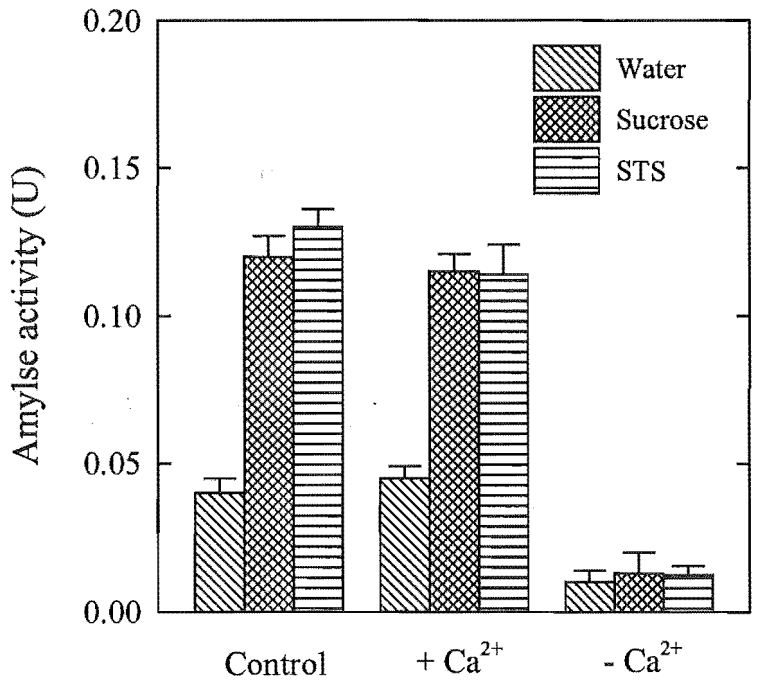


**Figure 3-45 Effect of heat treatment in the presence or absence of $Ca^{2+}$ on amylase activity in extracts of the reproductive parts of open gentian flower**

Extracts of the reproductive parts of open gentian flower from the stems pulsed for 24 h with water, 3% (w/v) sucrose or 0.5 mM STS were incubated with 0.2% soluble potato starch solution in the presence or absence of 10 mM $Ca^{2+}$ first at 70°C for 5 min and then at 37°C for 15 min. After this, amylase activity was measured immediately. The control assay reactions were carried out without heat treatment at 70°C for 5 min prior to incubation at 37°C for 15 min. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

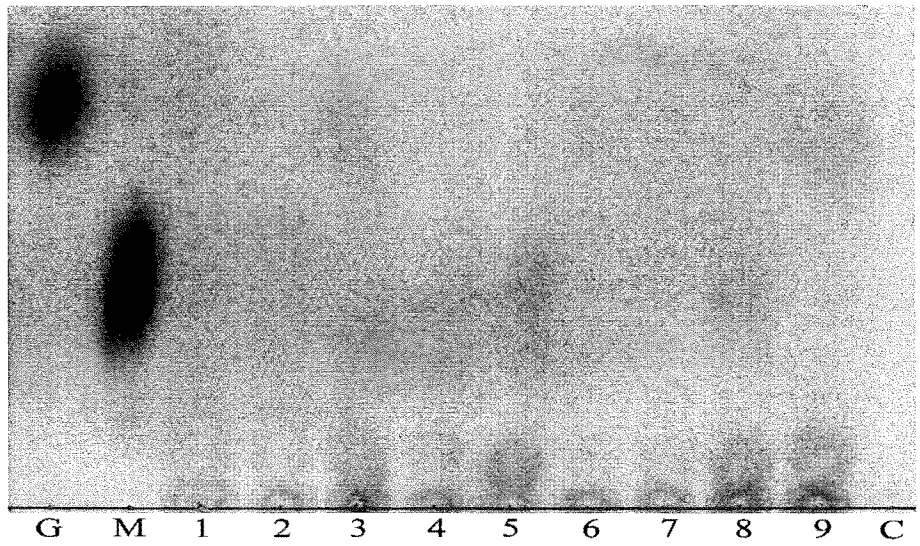


**Figure 3-46 Effect of incubation with EDTA on amylase activity in extracts of different gentian flower parts**

Extracts from different flower parts (lane #1 to lane # 9) were incubated with 0.2% soluble potato starch solution in the presence of 40 mM EDTA at 37°C for 15 min. 20 µl samples were taken and spotted on Whatman No 1 paper. Sugars were resolved in ethyl acetate-pyridine-$H_2O$ (12:4:3). G and M: 5 µl of 1% glucose and 5 µl of 1% maltose standards, respectively; 1, 2 and 3: petals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 4, 5 and 6: sepals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 7, 8 and 9: reproductive parts from the flower pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; C: 20 µl control without CE in the reaction.

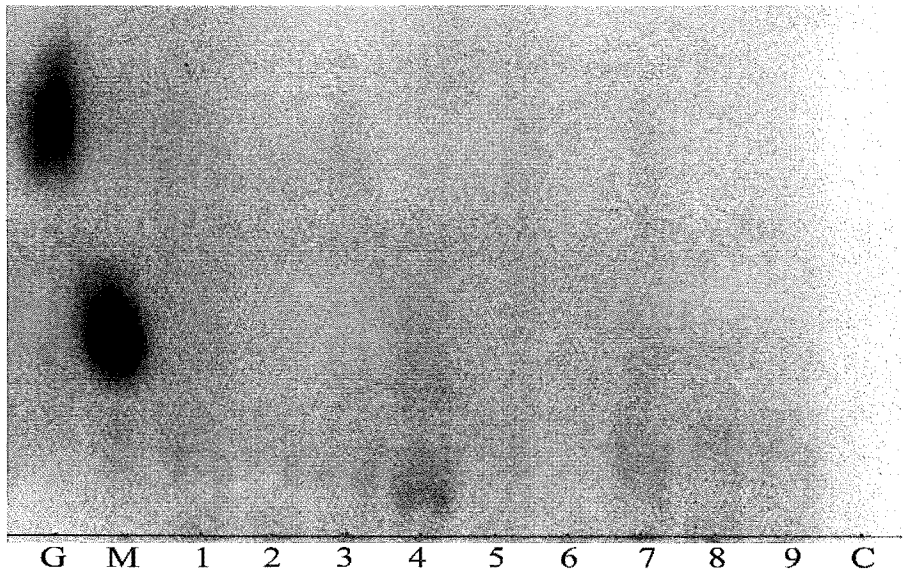


**Figure 3-47 Effect of incubation at pH 3.0 on amylase activity in extracts of different gentian flower parts**

Extracts from different flower parts (lane #1 to lane # 9) were incubated with 0.2% soluble potato starch solution at pH 3.0 at 37°C for 15 min. 20 µl samples were taken and spotted on Whatman No 1 paper. Sugars were resolved in ethyl acetate-pyridine-$H_2O$ (12:4:3). G and M: 5 µl of 1% glucose and 5 µl of 1% maltose standards, respectively; 1, 2 and 3: petals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 4, 5 and 6: sepals from the flowers pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; 7, 8 and 9: reproductive parts from the flower pulsed for 24 h with $H_2O$, 3% sucrose and 0.5 mM STS, respectively; C: 20 µl control without CE in the reaction.

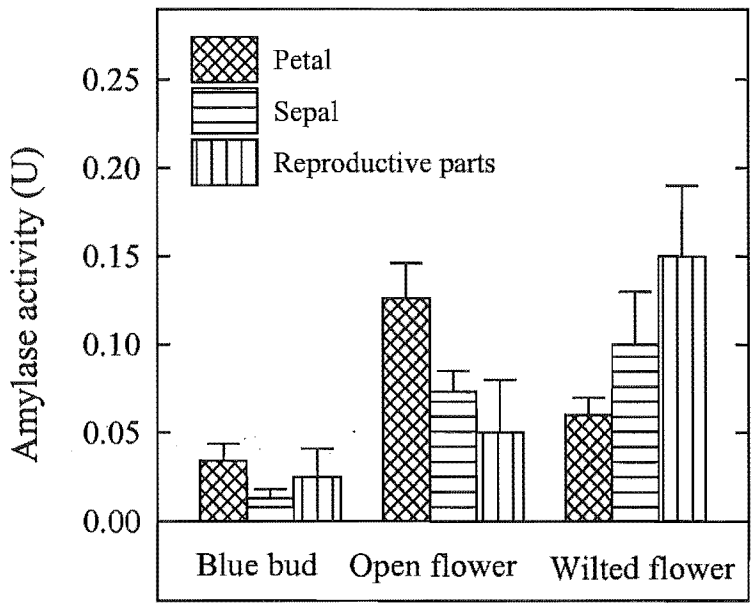


**Figure 3-48 Time course of α-amylase activity development in different flower parts of *G. triflora***

The extracts prepared from petal, sepal and reproductive parts of gentian flowers were first heated at 70°C for 5 min, and then incubated at 37°C for 15 min. Amylase activity was assayed from the different parts of gentian flowers at different life stages. One unit of enzyme activity (U) is defined as a change in 1 unit of absorbance at 620 nm. Values are means of 3 replicates ± SE.

## 3.3.5. *Protein Assay*

### 3.3.5.1 Total protein content in different developmental stages of gentian flowers

Total protein contents of the petal were obviously high at the blue bud stage but declined at the later stages (Table 3-11). In contrast, those in the sepal changed little. At a given developmental stage (except in the petal of wilted flowers) among the different flower parts, reproductive parts had the highest while sepals had the lowest protein level (Table 3-11).

### 3.3.5.2 Effect of pulsing treatments on total protein contents in different floral parts

In both the petal and reproductive parts of gentian flowers at the blue bud and open flower stages which were pulsed with sucrose (3%, w/v) or STS (0.5mM), the protein contents were higher than that in the control ($H_2O$ pulsed flowers) (Table 3-12 and Table 3-13). Pulsing of wilted flowers with sucrose or STS only led to an increase in the protein levels of the reproductive parts (Table 3-14). The pulsing treatments had no effect on the protein contents of the sepal at the 3 different development stages (Tables 3-12 to 3-14).

### 3.3.5.3 SDS-PAGE of proteins

Some differences were observed after SDS-PAGE of proteins from different gentian flower parts in response to sucrose or STS pulsing (Figure 3-49). In particular, a low molecular weight band with an apparent molecular weight of about 24 Kilo Dalton was found in extracts of the petal from flowers pulsed with 3% sucrose or 0.5 mM STS. This band was absent from the control petal extract and the other two floral parts in response to the various pulsing treatments.

**Table 3-11 Total protein contents of different parts of *G. triflora* flowers at 3 different developmental stages**

| Flower parts | Developmental Stages | Total protein contents (μg $gFW^{-1}$) |
|---|---|---|
| Petal | Blue Buds | 194.67 ± 8.51a |
| | Open flowers | 165.67 ± 9.71b |
| | Wilted flowers | 65.33 ± 9.02c |
| | | |
| Sepal | Blue Buds | 95.33 ± 5.51a |
| | Open flowers | 92.33 ± 4.51a |
| | Wilted flowers | 91.67 ± 3.51a |
| | | |
| Reproductive parts | Blue Buds | 223.33 ± 6.51a |
| | Open flowers | 186.67 ± 4.51b |
| | Wilted flowers | 177.00 ± 6.25b |

Values are means of 3 replicates ± SE. Data about a flower part marked by the same letter are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-12 Total protein contents in different parts of *G. triflora* flowers that were pulsed for 24 h with water, 3% sucrose or 0.5 mM STS at blue bud stage**

| Flower parts | Treatments | Total protein contents (μg $gFW^{-1}$) |
|---|---|---|
| Petal | $H_2O$ | 145.0 ± 2.13b |
| | Sucrose | 221.2 ± 3.10a |
| | STS | 220.0 ± 4.23a |
| | | |
| Sepal | $H_2O$ | 86.2 ± 3.61a |
| | Sucrose | 86.7 ± 2.28a |
| | STS | 89.7 ± 3.20a |
| | | |
| Reproductive parts | $H_2O$ | 148.8 ± 2.23b |
| | Sucrose | 234.2 ± 4.10a |
| | STS | 228.2 ± 4.33a |

Proteins were extracted from the different parts immediately after the various pulsing treatments. Values are means of 3 replicates ± SE. Data about a flower part marked by the same letter are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-13 Total protein contents in different parts of *G. triflora* open flowers that were pulsed for 24 h with water, 3% sucrose or 0.5 mM STS**

| Flower parts | Treatments | Total protein contents (μg $gFW^{-1}$) |
|---|---|---|
| Petal | $H_2O$ | 137.6 ± 2.21b |
| | Sucrose | 216.2 ± 2.99a |
| | STS | 224.0 ± 5.78a |
| | | |
| Sepal | $H_2O$ | 84.3 ± 3.53a |
| | Sucrose | 85.7 ± 4.48a |
| | STS | 86.3 ± 4.21a |
| | | |
| Reproductive parts | $H_2O$ | 141.8 ± 3.52b |
| | Sucrose | 214.3 ± 4.80a |
| | STS | 208.9 ± 4.26a |

Proteins were extracted from the different parts immediately after the various pulsing treatments. Values are means of 3 replicates ± SE. Data about a flower part marked by the same letter are not significantly different ($p < 0.05$) according to the Tukey's test.

**Table 3-14 Total protein contents in different parts of wilted *G. triflora* flowers that were pulsed for 24 h with water, 3% sucrose or 0.5 mM STS**

| Flower parts | Treatments | Total protein contents (μg $gFW^{-1}$) |
|---|---|---|
| Petal | $H_2O$ | 55.20 ± 5.02a |
| | Sucrose | 58.28 ± 3.53a |
| | STS | 59.02 ± 5.78a |
| | | |
| Sepal | $H_2O$ | 79.02 ± 3.12a |
| | Sucrose | 80.25 ± 3.28a |
| | STS | 80.60 ± 3.63a |
| | | |
| Reproductive parts | $H_2O$ | 125.8 ± 2.22b |
| | Sucrose | 180.6 ± 4.50a |
| | STS | 178.9 ± 4.20a |

Proteins were extracted from the different parts immediately after the various pulsing treatments. Values are means of 3 replicates ± SE. Data marked by the same letter in a column are not significantly different ($p < 0.05$) according to the Tukey's test.

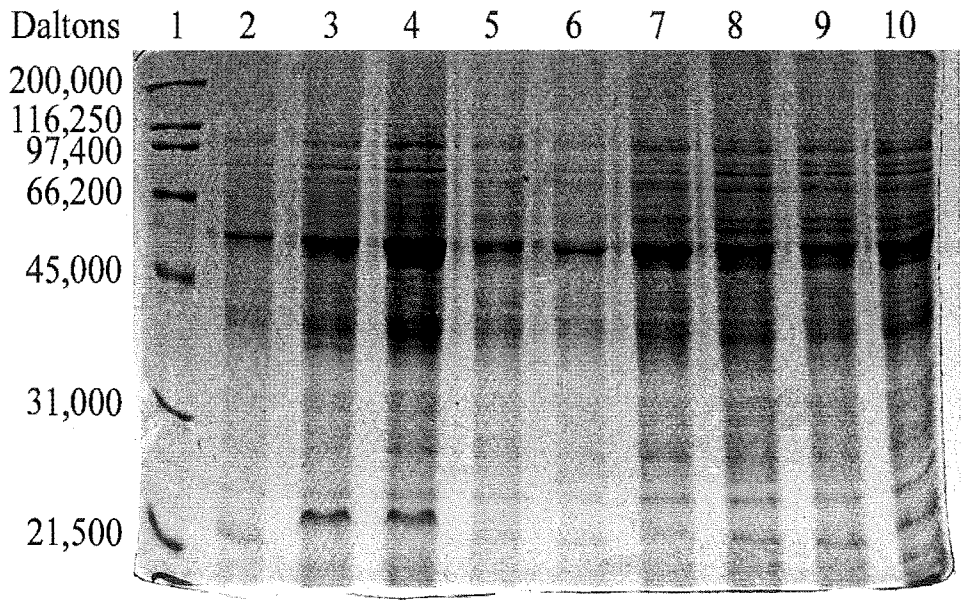


**Figure 3-49 SDS-PAGE of proteins extracted from different parts of *G. triflora* flowers at the open flower stage that were pulsed with water, 3% (w/v) sucrose or 0.5 mM STS**

Lane 1: the molecular weight standards; lanes 2-4: petal extracts from control (water pulsed), sucrose and STS pulsed flowers, respectively; lanes 5-7: sepal extracts from control, sucrose and STS pulsed flowers, respectively; lanes 8-10: extracts of reproductive parts from control, sucrose and STS pulsed flowers, respectively. 10 μl proteins were loaded in each of lanes.

### 3.3.6. *The isozymes of α-amylase*

To study if the differences in α-amylase activity following pulsing of different flower parts with 3% sucrose or 0.5 mM STS were due to different isozymes, isoelectric focusing of the extracts were first attempted before staining for amylase activity with $I_2$/KI. Despite numerous repeated attempts, the resolution obtained was far from being satisfactory although it seems that there were many isoenzymes present in the extract of different *G. triflora* flower parts in response to the different pulsing treatments (Appendix E). Satisfactory resolution was obtained on a native PAGE gel at pH 8.8 (Figure 3-50). The extracts of the different flower parts in response to the different pulsing treatments all seemed to have the same predominant isoform of amylase activity. It also appears that the sepal had lower amylase activity than the other 2 flower parts.

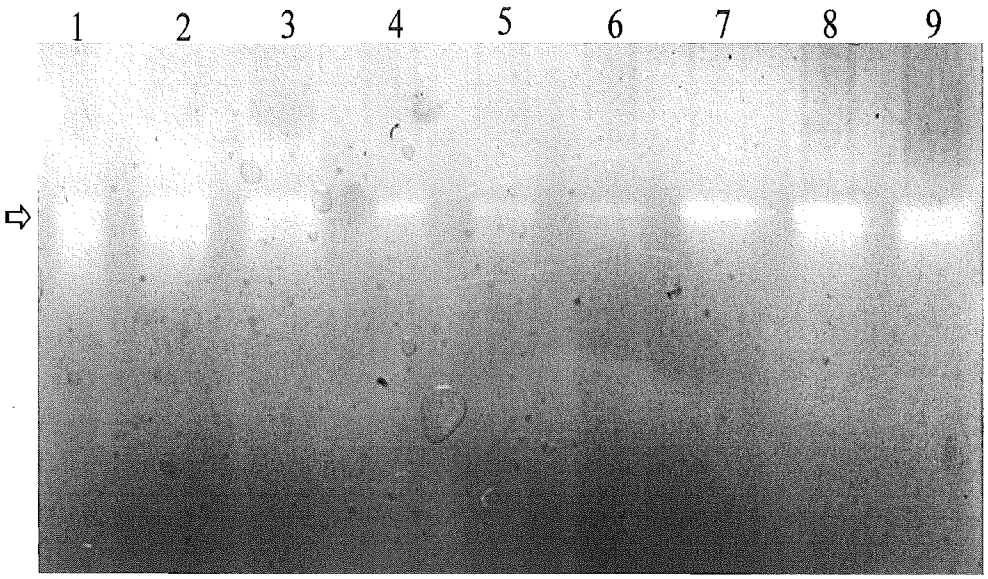


**Figure 3-50 Native PAGE showing amylase activity in the extracts of different parts of open *G. triflora* flowers after 24 h of pulsing with water, 3% (w/v) sucrose or 0.5 mM STS**

Lanes1-3: extracts of petal from flowers pulsed with water, sucrose and STS, respectively; lanes 4-6: extracts of sepal from flowers treated with water, sucrose and STS, respectively; lanes 7-9: extracts of reproductive parts from flowers treated by water, sucrose and STS, respectively. Arrow indicates the position of amylase activity band.

## 3.4. ACC oxidase gene expresion – A preliminary molecular biology study

### 3.4.1. *Total RNA isolation*

Total RNAs were isolated from water-, sucrose- and STS-treated flower petals of *G. triflora* using the method described in section 2.5.2.1 (Figure 3-51).

### 3.4.2. *Quantification of the isolated total RNA*

Quantification of the total RNA was performed using a spectrophotometer (LKB Biochrom Ultrospec® Plus 4054 UV/Visible Spectrophotometer). The results indicated that the total RNAs extracted from the petal of *G. triflora* flowers after water, sucrose or STS pulsing had $A_{260/280}$ ratios of 1.604, 1.416 and 1.473, respectively.

### 3.4.3. *Optimization of RT-PCR*

The protocol for running the RT-PCR was optimized with different concentrations of template RNA extracted from the petal of open gentian flower using a pair of primers to amplify the likely conserved region (~ 800 bp) of ACC oxidase genes from plants. The template RNA concentrations tested ranged from 0.4 to 1.2 μg $\mu l^{-1}$. The results showed, from 0.7 % agarose gel electrophoresis (Figure 3-52), that when the higher concentration of the template RNA was used, the sharper the band was resulted. The addition of 1.2 μg template RNA gave the best result with a clear band of higher molecular weight (931 bp) in addition to the lower molecular weight band which was also present in the RT-PCR reaction mixture without template RNA added (Figure 3-52).

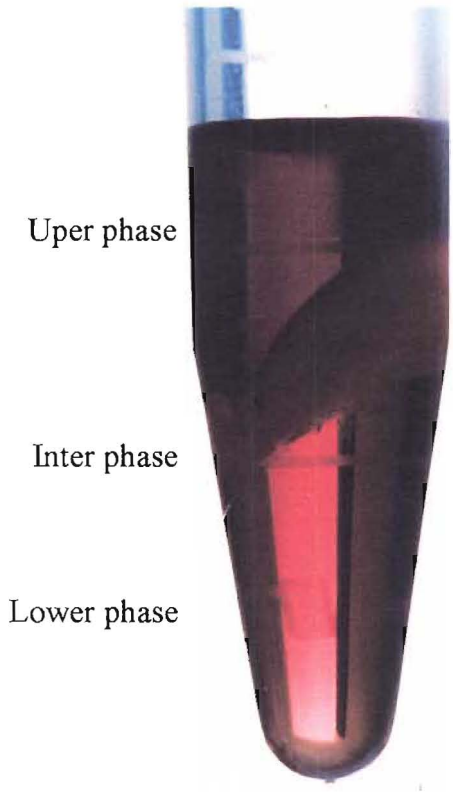


**Figure 3-51 RNA isolated from the petal of open *G. triflora in vitro* flower**

The mixture in the Eppendorf tube was separated into a lower red, phenol-chloroform phase, a white organic middle-phase, and a purple upper aqueous phase. RNA remained exclusively in the aqueous phase.

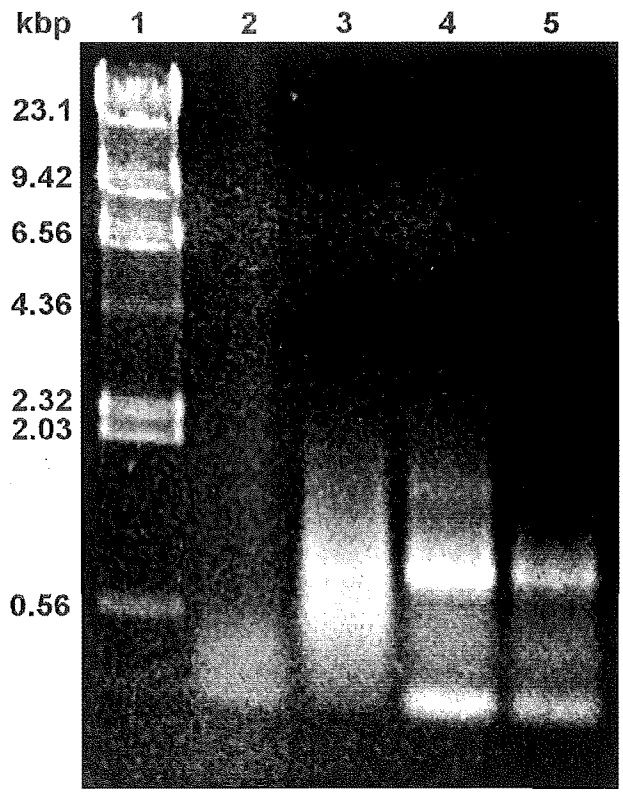


**Figure 3-52 Optimization of RT-PCR with regard to different concentrations of template RNA extracted from the petal of open *G. triflora in vitro* flower**

After RT-PCR, 10 μl the reaction mixture was separated using 0.7% (w/v) agarose gel electrophoresis. Lanes are as follows: 1. DNA molecular weight standards; 2. Control (without RNA template); 3. Template RNA concentration at 0.4 μg $\mu l^{-1}$; 4. Template RNA concentration at 0.8μg $\mu l^{-1}$; 5. Templates RNA concentration at 1.2 μg $\mu l^{-1}$.

Few RT-PCR protocols were tried with RNA templates from gentian before the initiation of our experiments. Optimisation of the RT-PCR was therefore attempted according to the suggestions by Dr Leung (David Leung, personal communication, University of Canterbury, Christchurch, New Zealand). Apart from the tests for the optimisations described in this experiment, e.g. the concentrations of RNA template and RNase inhibitor, some other factors were also thought to be critical for running a RT-PCR. These include suitable primers being designed and the annealing temperature in the RT-PCR reaction. Primers for the ACC oxidase (ACO) fragments were carefully designed and the temperature of elongation calculated according to the content of CG in the primer. The sequences of primers are AO1: 5'-GATGCTTGTGAGAACTGGGG-3' and AO2: 5'-GCTTCAAATCTTGGCTCCTT-3'. The elongation temperature ($t$) was then calculated according the contents of the primers as in the following equation:

$$t = 4\,(G+C) + 2\,(A+T)$$

(Dr. Leung, University of Canterbury, New Zealand, personal communication)

therefore,

$$t_{AO1} = 4\,(9+2) + 2\,(4+5) = 62°C$$

$$t_{AO2} = 4\,(3+6) + 2\,(3+8) = 58°C$$

$$t = (62 + 58) / 2 = 60°C$$

This temperature was used for annealing of the RT-PCR instead of 50°C which was used in other suggestions. This temperature greatly improved the efficiency of the RT-PCR and the expected RT-PCR product was easily detectable on 1% agarose gel even when the volume of the product analyzed was as low as 5 μl (data not shown).

### 3.4.4. *Recovery of DNA from low temperature melting (LTM) agarose*

After RT-PCR using RNA extracted from the petal of open *G. triflora in vitro* flower as template, the reaction mixture was electrophoresed on a 1% (w/v) LTM agarose gel to recover the 931 bp product. As shown on 1% LTM agarose gel (Figure 3-53), the recovered DNA appeared as a very faint band and the concentration was about 0.105 $\mu g\ \mu l^{-1}$.

To avoid the interference of free DIG-11-dUTP in the later step of hybridization and detection, RT-PCR-synthesized-DNA and later the DIG-labelled DNA were proposed to be purified by agarose gels. A couple of DNA recovery techniques were put in trials including the "freeze-squeeze" method which did not work. The method of "low melting temperature agarose gel" resulted in a very little recovered DNA which could not be quantitatively determined required for DNA amplification, DNA labelling and RNA hybridization. Therefore, an alternative method had to be generated.

### 3.4.5. *Amplification of cDNA directly from RT-PCR products*

After RT-PCR, the reaction mixture was used as a template for direct amplification via PCR using Taq DNA polymerase. 1, 5 and 10 μg of the RT-PCR product were tested. The highest concentration used gave a long smear with a faint band of the expected size (Figure 3-54). The lower concentrations gave better results with less smear and clearer bands.

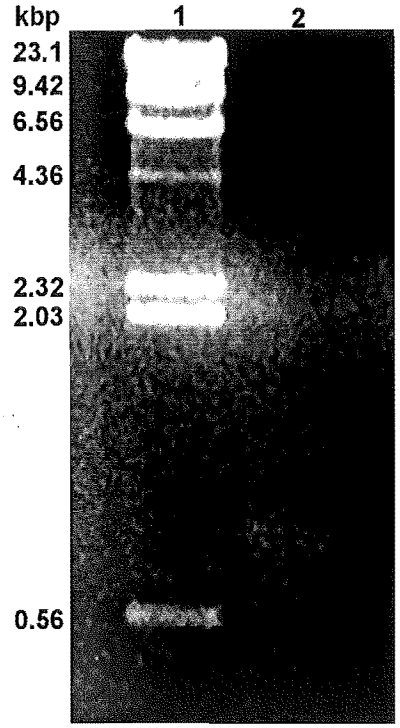


**Figure 3-53 RT-PCR product recovered and separated on 1% (w/v) LTM agarose**

After RT-PCR using RNA extracted from the petal of open *G. triflora in vitro* flower as template, the reaction mixture was electrophoresed on 1% (w/v) LTM agarose gel to recover and separate the 931 bp product. Lane1: DNA molecular weight standards, 5 μl loaded; lane 2: the 931 bp RT-PCR product, 10 μl loaded.

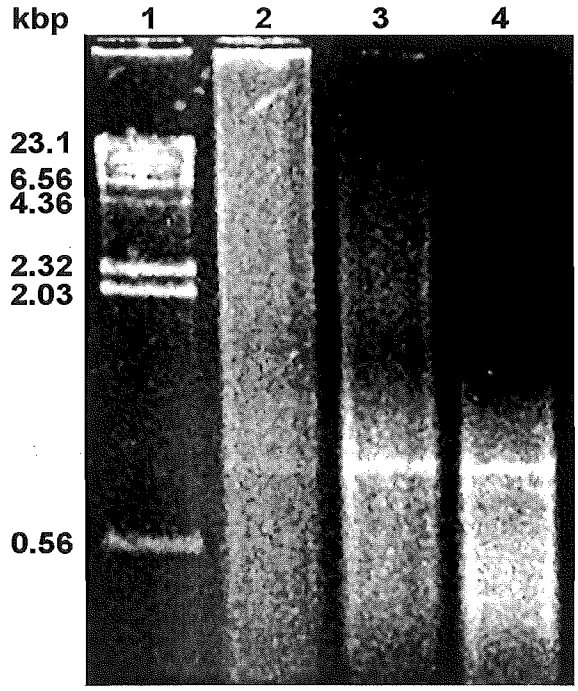


**Figure 3-54 Amplification of cDNA directly from the recovered RT-PCR product**

After PCR amplification, aliquotes of the reaction mixture were electrophoresed on 2% (w/v) agarose. Lanes 1: DNA molecular weight standards, 5 µl loaded; lane 2: 10 µl template; lane 3: 5 µl template; lane 4: 1 µl template.

### 3.4.6. *PCR-DIG labelling of DNA*

DNA was labelled via PCR incorporating the non-radioactive DIG tags. The results of using Klenow enzyme or Taq polymerase in the PCR reactions were compared (Figure 3-55). A much stronger band of interest (the 931 basepairs product) resulted when Taq polymerase rather than Klenow enzyme was used. This was confirmed when the labelling efficiency of DIG-DNA was estimated after DNA dot blotting (Figure 3-56; also see Table F1 and F2 in Appendix F).

### 3.4.7. *Change of ACC oxidase-related transcripts in response to sucrose or STS pulsing of gentian flowers*

RNA dot blots were prepared by dot blotting 25 ng of total RNA onto nylon membrane and hybridization was carried out with the DIG-labelled PCR-generated fragment of ACC oxidase gene as the probe. The hybridization signal was stronger in the water control than in the other two pulsing treatments, i.e. sucrose- or STS-treated samples using the probes generated with both the Taq polymerase and the Klenow enzyme (Figure 3-57).

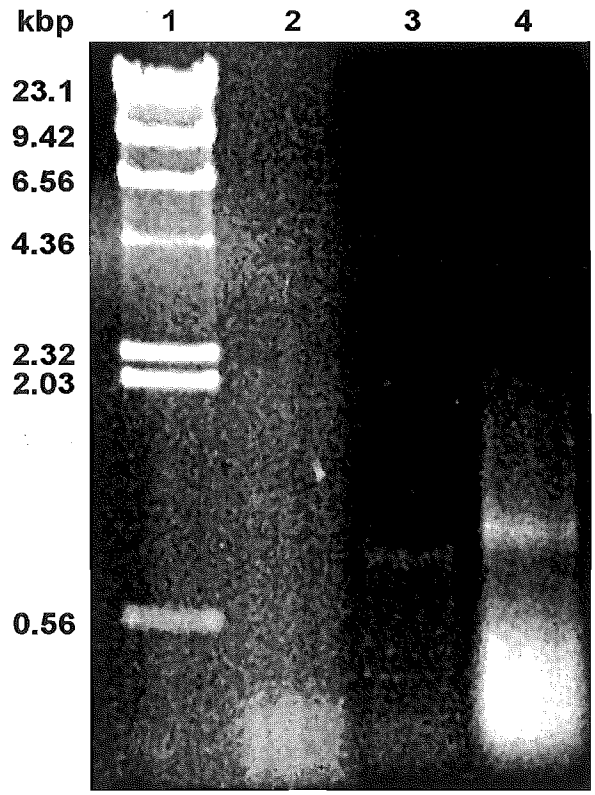


**Figure 3-55 PCR-assisted digoxigenin (DIG)-labelling of the reaction product from a previous RT-PCR using RNA extracted from the petal of open *G. triflora* flower as the template**

After PCR, aliquot of the reaction mixtures was analyzed using 1% agarose gel electrophoresis. Lane 1: DNA molecular weight standards, 5 μl loaded; lane 2: control (without DNA template in the reaction), 10 μl loaded; lane 3: reaction with Klenow enzyme, 10 μl loaded; lane 4: reaction with Taq polymerase, 10 μl loaded.

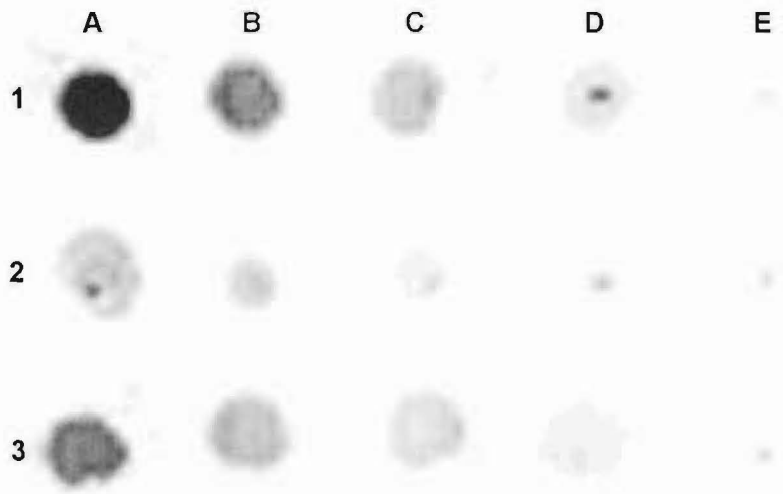


**Figure 3-56 Estimating the yield of DIG-labelled DNA on a dot blot**

The yield of DIG-labelled DNA from the same experiment as described in Figure 3-55 was estimated after dot blotting. Rows show the different dilutions of 1: control DNA; 2: PCR product from Klenow enzyme reaction; 3: PCR product from Taq polymerase reaction. Concentrations of DIG-labelled control DNA: A. 100 pg $\mu l^{-1}$; B. 10 pg $\mu l^{-1}$; C. 1 pg $\mu l^{-1}$; D. 0.1 pg $\mu l^{-1}$ and E. 0.01 pg $\mu l^{-1}$; Columns A-E of rows 2 and 3 show the effects of different dilutions, A. 1:10; B. 1:100; C. 1:1000; D.1:10000 and E. 1:100000 of DIG-labelled DNA using Klenow enzyme or Taq polymerase, respectively.

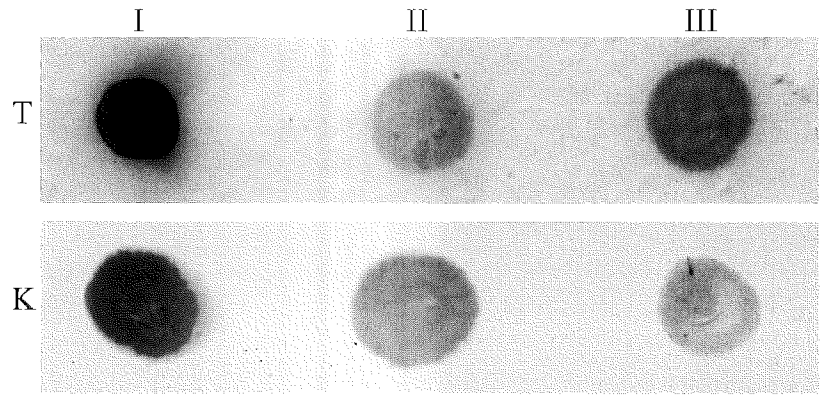


**Figure 3-57 Dot blot analysis of total RNA extracted from petals of open *G. triflora* flowers**

One μl (containing 25 ng) total RNA was dotted onto each spot of the nylon membrane. Spots in row, T and K are results from hybridization with DIG-labelled DNA via PCR using Taq polymerase and Klenow enzyme, respectively, as the probes at the concentrations of 25 ng $ml^{-1}$ for the former and 10 ng $ml^{-1}$ for the latter. Columus I – III are RNA extracted from control (water pulsed flower petals); 3% sucrose and 0.5 mM STS pulsed flower petals, respectively.

# Chapter 4. Discussion

## 4.1. Factors affecting *in vitro* flowering

The general body plan of plants is established during embryogenesis. However, much of plant development occurs postembryonically, through the reiterative production of organ primordia at the shoot apical meristem (SAM). In most species, the SAM initially gives rise to vegetative organs such as leaves, but at some point the SAM makes the transition to reproductive development and the production of flowers. This change in the developmental fate of primordia initiated at the SAM is controlled by environmental and endogenous signals (Bernier, 1988; McDaniel *et al.*, 1992). In some species, the timing of flowering is primarily influenced by environmental factors, which serve to communicate the time of year and/or growth conditions favourable for sexual reproduction and seed maturation. These factors include photoperiod, light quality, light quantity, vernalisation, and nutrient and water availability. Other species are less sensitive to environmental variables and appear to flower in response to internal cues such as plant size or number of vegetative nodes. Flowering can also be induced by stresses such as nutrient deficiency, drought, and overcrowding. This response enables the plant to produce seeds, which are much more likely to survive the stress than is the plant itself (Levy and Dean, 1998). In the present study, the induction of flowering in *G. triflora* shoot was also shown to be controlled by various factors, such as carbohydrates, plant growth regulators, pH and light intensity.

Indeed, the ability of explants to form flowers *in vitro* depends on numerous factors, internal and external, chemical and physical, and virtually all of these factors interact

in various complex and unpredictable ways (Tran Thanh Van, 1973; Lang, 1987; Jumin and Ahmad, 1999). The classical experiment conducted by Tran Thanh Van (1973) showed that *in vitro* flowering could form from cultures of non-meristematic tissues by using thin cell layers of *Nicotiana tobaccum* floral shoots. Research has shown that cultures of other plant tissues such as tendrils, roots, leaf discs, thin sections from stem and inflorescence axis, micro-inflorescence and isolated protoplasts can also result in flower formation *in vitro* (Liu and Li, 1989). In the present study, *in vitro* flowering could occur from the nodal explant cultures of *G. triflora*. The flowering rate decreased with increased distance from the apex, indicating the presence of a floral gradient in the shoot developed from nodal sections. Poor flowering formation in older tissues might be related to the presence of floral inhibitors. Conversely more floral promoters might be present in younger tissues. The existence of a floral gradient *in vitro* has been reported with several species and explants sources (Scorza, 1982; Compton and Vielleux, 1992). This apex to base floral gradient may also reflect changes in the capacity of cells to enter into a reproductive state in the course of ontogeny (Scorza and Janick, 1980 and McDaniel *et al.*, 1989).

Over the years, physiological studies have led to three models for the control of flowering (Bernier, 1988; Thomas and Vince-Prue, 1997): (1) The florigen concept. This is based on the transmissibility of substances or signals across grafts between reproductive "donor" shoots and vegetative "recipients." It was proposed that florigen, a flower-promoting hormone, was produced in leaves under favourable photoperiods and transported to the shoot apex in the phloem. (2) The nutrient diversion hypothesis. This model proposed that inductive treatments result in an increase in the amount of assimilates moving to the apical meristem, which in turn induces flowering. (3) The multifactorial control model. This model proposed that a number of promoters and inhibitors, including phytohormones and assimilates, are involved in controlling the developmental transition. According to this model, flowering can only occur when the limiting factors are present at the apex in the appropriate concentrations and at the right times. This model attempted to account for the diversity of flowering responses by proposing that different factors could be limiting for flowering in different genetic background and/or under particular environmental conditions. The view that

assimilate is the only important component in directing the transition to flowering is superseded by the third model. Thus it is generally believed that, based on considerable physiological analyses, certain compounds including plant growth regulators and carbohydrates are implicated in controlling flower formation.

### 4.1.1. *The role of plant growth regulators in controlling flowering*

Numerous data showed that the plant growth regulator requirements of plants for *in vitro* flowering are variable. The growth and development of flower buds in some monocots (Mohanram and Batra, 1970; Zhong *et al.*, 1992) and dicots (Rastogi and Sawhney, 1987; Narasimhulu and Reddy, 1984) seem to require a cytokinin. Some plants require a cytokinin in combination with either an auxin (Peeters *et al.*, 1994), gibberellic acid (Rastogi and Sawhney, 1987) or a combination of $GA_3$ and IAA (Tepfer *et al.*, 1966).

The role of gibberellins in the transition to flowering has been difficult to establish (Levy, 1998). On one hand, there are many examples in which the abundance or composition of endogenous gibberellins changes under conditions that induce flowering (Pharis and King, 1985). Furthermore, because applying certain gibberellins can induce flowering in some species, there has been an emphasis on the study of gibberellins in floral initiation and in the search for florigen (Chouard, 1960; Evans, 1971; Zeevaart, 1983; Thomas and Vince-Prue, 1997). On the other hand, applied gibberellins are rarely effective at inducing flowering in short-day plants. Moreover, they generally inhibit flowering of woody angiosperms, although they do promote flowering of conifers (Pharis and King, 1985). Even with long-day plants, the same gibberellin can have a different effect in different species. For example, 2,2-dimethyl $GA_4$ has potent florigenic activity when applied to *Lolium temulentum* but has no effect on flowering in *S. alba* (Bernier *et al.*, 1993).

Gibberellic acid ($GA_3$) as a plant hormone effects shoot elongation, seed germination and flowering in long-day plants. Some plants, such as cabbages and carrots, form

rosettes before flowering. In these plants, flowering can be induced by exposure to long days, to cold, or to both. Following the appropriate exposure, the stems elongate and flowering occurs. Application of $GA_3$ to such plant causes bolting and flowering without appropriate cold or long-day exposure. Foliar application of $GA_3$ in a dioecious cucurbit *Trichosanthes dioica* could increase the number of female flowers resulting in increased fruit-setting (Basu *et al.*, 1999). Increased $GA_3$ concentration (250 to 2000 mg $l^{-1}$) resulted in increased flower number in *Syngonium podophyllum* (Henny *et al.*, 1999). Similar results were also obtained in some *in vitro* plant cultures. $GA_3$ supplemented in the culture medium induced flowering from explants of *Pisum sativum* L (Franklin *et al.*, 2000) and *Chenopodium murale* L (Mitrovic *et al.*, 2000). It is also believed that $GA_3$ supplemented in the culture medium or synthesised in the flower itself may be responsible for *in vitro* seed setting of green pea (Franklin *et al.*, 2000). One of the ways through which $GA_3$ might affect flower formation and pollen development is through the availability of soluble sugars because it induces synthesis of amylase which breaks down starch, and provides free sugars for the developing organ (Sawhney and Rastogi, 1990).

However, apart from the induction of multiple long shoots, the application of $GA_3$ in the medium had no effect in inducing *in vitro* flowering of *G. triflora* on given condition in the present study. This might be because $GA_3$ could only affect shoot growth of *G. triflora*. Kostenyuk *et al* (1999) reported that gibberellic acid markedly delayed *in vitro* flowering in *Cymbidium niveo-marginatum* even when the flower-promoting treatment was applied. Gibberellins applied *in vivo* promote flowering of some species and inhibit flowering of others (Evans, 1971). The effects of gibberellins are evident *in vitro* where they generally inhibit floral and vegetative bud initiation in some species but could promote flowering of explants in others (Chang and Hsing, 1980). Lang (1965) hypothesized that gibberellins affect floral development rather than induce flowers. This hypothesis was supported by the facts that $GA_3$ inhibits *in vitro* bud formation but induces "bolting" when applied directly to flower buds *in vitro* (Wardell and Skoog, 1969; Scorza, 1979).

Gibberellins are not the only class of phytohormones that has been implicated in affecting flower formation. It has been postulated that the flowering stimulus which may consist of cytokinin to trigger the initial mitotic event that precedes flowering (Bernier *et al.*, 1977; Havelange *et al.*, 1986), plus at least one other factor. For example, there is evidence from studies on *S. alba* that long-distance signalling by cytokinins might paly a role in flowering in response to inductive photoperiods (Bernier *et al.*, 1993). Inductive photoperiods cause the rapid and transient export of sucrose from the leaves to both the shoot and root meristems. In the root, this is followed by the export of cytokinin, primarily zeatin riboside, to the shoot and leaves, presumably via the xylem. Subsequently, another cytokinin, isopentenyladenine riboside, moves out of the leaves, and some of which makes its way to the shoot apex, where its levels increase within 16 hours of induction (Bernier *et al.*, 1993).

The data in the present study supports the theory of a multiple-factorial control of flowering (Evans, 1969; Bernier *et al.*, 1993). The results showed that *in vitro* flower and shoot formation in *G. triflora* differ in the requirement for light intensities at similar BA concentrations. Shoot production was evidently good at the low PPFD (30 mol $m^{-2}$ $s^{-1}$) over a range of BA concentrations (0.2 – 1.0 mg $l^{-1}$). Prolific flowering occurred the best at PPFD of 60 mol $m^{-2}$ $s^{-1}$ when supplied with BA at 0.2 mg $l^{-1}$.

In addition to giberellins and cytokinins, other phytohormones, such as polyamines, auxins, abscisic acid (ABA) and ethylene, may be involved in flowering under certain circumstances and in some species (Maritinez-Zapater *et al.*, 1994). In *Arabidopsis*, the ethylene-insensitive mutant *ein2* is slightly delayed in flowering, and ABA-deficient mutants flower somewhat early under noninductive photoperiods (Maritinez-Zapater *et al.*, 1994), suggesting a role for ethylene and ABA in floral promotion and repression, respectively. Polyamines play various roles in plant development including flower formation (Evans and Malmberg, 1989). Dramatic results have been reported with the floral thin-layer (FTL) system in tobacco (Tran Thanh Van, 1973). Kaur-Sawhney *et al.* (1988) showed that spermidine levels increased in tobacco FTL explants cultured on medium for flower bud induction and the addition of spermidine into medium for vegetative bud induction caused the appearance of a number of floral

buds on the explants. Supporting evidence for a role of polyamines in the control of the floral transition has also come from the use of inhibitors of spermidine and spermine synthesis. For example, inhibition of polyamine synthesis by various inhibitors deceased or delayed flowering in *Speirodela punctata*, tobacco and chrysanthemum (Bendeck de Cantu and Kandeler, 1989; Burtin *et al.*, 1991; Aribaud and Maritn-Tanguy, 1994). Bais *et al.* (2000) found that the morphogenetic response and the endogenous conjugated pool of polyamines were diminished in polyamine inhibitors (DFMA and DFMO) treatments, but could be restored by addition of putrescine and $AgNO_3$ on shoot multiplication and *in vitro* flowering of *Cichorium intybus* L. cv. Lucknow local. These findings suggest that polyamines may be causally related to flowering, and that conjugated polyamines may constitute potential markers for floral initiation (Daoudi and Bonnet, 1998). Unfortunately, the present study did not test if these phytohormones could effect floral formation of *G. triflora* due to the limited time and funds. However, it could be an interesting aspect to be investigated for *in vitro* flowering of gentians in future.

### 4.1.2. *The role of sugars in flower formation*

In addition to plant growth regulators such as gibberellins and cytokinins, sugars could also play an important role in regulation of flower formation.

As shown in the present work, the shoot from *G. triflora* nodal explants flowered in the medium containing sucrose under light condition. Furthermore, the results indicated that there was an intereaction between sucrose concentration and light intensity. This has also been demonstrated recently, for example, in orange jessamine (Jumin and Nito, 1996) and Alstroemeria (Kristiansen *et al.*, 1999).

In the present study, prolific flowering occurred the best at 60 mol $m^{-2}$ $s^{-1}$ PPFD and 3% (w/v) sucrose. At this sucrose concentration, the high PPFD (120 mol $m^{-2}$ $s^{-1}$) inhibited flower and shoot formation while the low PPFD (30 mol $m^{-2}$ $s^{-1}$) was not enough to stimulate prolific flowering although it was the best in inducing shoot proliferation *in vitro*. The results of the present study were similar to those obtained

by Dickens and van Staden (1988). They examined the effects of varying sucrose concentrations on *in vitro* floral induction in *Kalanchoe blossfeldiana*. It was found that sugar concentrations of 2% and 4% (w/v) were stimulatory to flowering, while a concentration above 4% became inhibitory. The effect of sugar concentration on *in vitro* flowering of *Tornenia* was also considered by Tanimoto and Harada (1981). It was found that high rates of floral bud formation occurred between 2% and 8% (w/v) sucrose. They also found that the mean number of floral buds per explant increased along with increasing sucrose concentrations. This contrasts with the findings of the present study, suggesting that different *in vitro* shoot culture systems might have different requirement for sucrose concentration as fas as floral initiation is concerned.

Comparing with shoot formation, where 2% sucrose was the most effective, a higher concentration of sucrose at 3% was required for *in vitro* flowering of *G. triflora*. It has been argued that high concentrations of carbohydrates within the media induce *in vitro* flowering due to osmotic potential effects rather than the accumulation of sufficient carbohydrates by the plant. However, this has been disproved with the work on *Zea mays* cv. Oh43 which requires 10% (w/v) sucrose media for optimal flowering (Pareddy and Greyson, 1989). In plant tissue, it was found that the endogenous level of free sugars in the apex increases dramatically during induction to flower (Bodson, 1977). These effects are not due to altered osmotic values but may be the result of enhanced activity of the pentose phosphate pathway in response to an excess of soluble sugars (Nitsch and Nitsch, 1967; Friend *et al.*, 1984; Bernier *et al.*, 1993).

### 4.1.3. *Other factors involved in in vitro flowering*

Rastogi and Sawhney (1987) observed that the *in vitro* growth and development of tomato flowers from sepal primordia stage to maturity was sensitive to, and regulated by, various factors such as cytokinin, auxin, gibberellin, sucrose and pH of the culture media. It was reported recently that the pH of media could influence *in vitro* flowering in orange jessamine (Jumin and Ahmad, 1999) and bamboo (John and Nadgauda, 1999). In the present study, the effect of varying pH of the medium on *in vitro* flowering under different light intensities was investigated. There is revealed an

interaction between pH and light intensity. The reason for this is not readily apparent. However, it might be related to the uptake of ingredients from the medium and their utilization. It could also be argued that at the low pH (3.7 and 4.7) there was some physical effect on the gelling of the media which might affect the response of the explants. However, using the same liquid media at the three pH values the same results in terms of shoot production and flower formation were obtained as when the media were gelled with agar (data not shown).

### 4.1.4. *Similarities of in vitro and in vivo flowers*

There is a high degree of similarity between *in vitro* and *in vivo* flowers of *G. triflora*. Most flowers were normal and complex, and appeared in the leaf axils or the top of the shoot, but malformations of the flowers were also observed on *in vitro* flowers of *G. triflora*. This may have been at least partially due to competition and/or nutritional deficiencies. Fresh subculture medium may be necessary to eliminate the nutritional deficiency during flower development. More work is needed to substantiate this possibility.

The pollen grains from both sources of *in vitro* and *in vivo G. triflora* flowers were observed using scanning electron microscope (SEM). The results showed that they are very similar in terms of shape and size although there were some undeveloped (malformed) pollen grains from *in vitro* flowers. This may be due to nutritional deficiencies. The pollen grains from both *in vitro* and *in vivo* sources were over 90% viable, as examined using the Alexander's procedure, indicating their potential for pollination. Germination test on the pollen grains revealed that both *in vitro* and *in vivo* pollen grains of *G. triflora* could germinate on controlled conditions, even the germination rate was lower in *in vitro* pollen grains compared to its *in vivo* counterparts. Nevertheless, these results further suggest the possibility of *in vitro* fertilization in *G. triflora*.

Seed formation was greatly improved by hand-pollination from 0.3% to 3% in the farm although it is still very low. After hand-pollination, no seed formation resulted from *in*

*vitro* flowers but only seed-like structures were observed. These were likely to be those unfertilized ovules of *G. triflora* and can be observed from both sources of the flowers. Higher seed set *in vitro* requires more research in the future.

## 4.2. Senescence of cut gentian flowers

Senescence and death are important processes in the life cycle of an organism: they occur at many stages during the development of an organism and at many levels (Leopold 1961, Noodén 1988). Death may occur selectively in a few cells, in specific organs, e.g. flower petals, or in a whole organism. Death is often the end result of internally programmed degeneration termed senescence (Noodén *et al.*, 1997). In flower petals, the often rapid and synchronous programmed death of the cells provides a model system to study molecular aspects of organ senescence (Rubinstein, 2000). In plants, senescence is considered to be internally programmed because it is specific and orderly in terms of when, where and how it occurs (Noodén and Leopold, 1978).

In the present study, wilting of cut gentian flowers is the visible sign of senescence. This is generally recognised as the petal cells lose turgor. Loss of turgor is presumably associated with deterioration of the cell membrane function, which is an early and characteristic feature of plant senescence engendering increased permeability, loss of ionic gradients, and decreased function of key membrane proteins such as ion pump (Taiz and Zeiger, 1998). In carnation, during petal senescence marked changes occurred in the water status of the tissue, including losses in water potential and turgor (Eze *et al.* 1986). The decline in membrane structural integrity at the onset of senescence appears to be largely attributable to accelerated metabolism of membrane lipids and ensuing change in the molecular organisation of the bilayer. Loss of membrane phospholipid is one of the best documented indices of membrane lipid metabolism during senescence and has been shown to occur during senescence of flower petals, leaves, cotyledons and ripening of fruits (Borochov *et al.*, 1982). This involves phase separation, which is a change in membrane lipid composition leading to leaky membranes and loss of ion gradients. Phase separation of the lipid bilayers of

cell membranes does not occur until the final stages of cell disassembly (Thomas *et al.*, 1997, 1998).

Although senescence does progress with age, it is not simply a passive aging process. It is controlled by internal and external signals, and it can be delayed or accelerated by altering these signals (Noodén 1988). Furthermore, senescence is an active process and is controlled by the nucleus (Noodén and Leopold, 1978; Thomas, 1987; Smart, 1994; Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997; McGrath and Ecker, 1998; Gershon and Gershon, 2000; Fujiki *et al.*, 2001).

### 4.2.1. *Regulation of flower senescence: ethylene and carbohydrate manipulations*

The most important hormones that regulate senescence in higher plants are cytokinins and ethylene. Cytokinins delay senescence in many plant species. The effects of cytokinin on metabolic processes include delays in the degradation of Chlorophyll and protein (Richmond and Lang, 1957; Rodoni *et al.*, 1998) concomitant with decreased activities of chlorophyllase (Sabter and Rodriguez, 1978) in senescing barley leaves. In contrast to cytokinin, ethylene hastens many metabolic processes of senescence (Brady, 1987). For example, in transgenic tomato, fruit ripening can be delayed by antisense expression of either ACC synthase (Oeller *et al.*, 1991) or ACC oxidase (Picton *et al.*, 1993), the two last enzymes in ethylene biosynthesis.

Ethylene is a pleiotropic plant growth modulator which is known to initiate many developmentally coordinated programs such as petal senescence (Halevy and Mayak, 1981) and fruit ripening (Tucker and Laties, 1984). Concentrations of ethylene are known to increase to high levels during senescence of cut flowers (Eze *et al.*, 1986). The data obtained from the present study showed that the petals of *G. triflora* produced ethylene in the course of senescence, which could be hastened by exogenous ethylene. There is growing evidence that in many cases ethylene exposure results in autocatalytic ethylene production and gene expression (Woodson & Lawton, 1988;

Broglie *et al.*, 1986). The results presented here indicate that exogenous ethylene could induce autocatalytic ethylene production in gentian petals and resulted in shortened flower vase life. This may imply that ethylene exposure could increase the development of petal cell sensitivity to the low basal level of endogenous ethylene. Moreover, like many ethylene-sensitive flowers, gentian flowers responded well to a post-harvest STS treatment. STS is a well-known inhibitor of ethylene action (Mayak *et al.*, 1973; Veen, 1979; Paulin and Jamain, 1982; Koyama and Uda, 1994; Kuiper *et al.*, 1995; Ichimura, 1998; Han, 1998).

Moreover, the present study shows that sucrose has a similar effect to STS as far as inhibiting ethylene production by gentian petals is concerned. The pulsing solutions containing either STS or sucrose has an anti-ethylene effect, which is likely to contribute to its effectiveness in extending the vase life of cut gentian flowers. It is perhaps not surprising that sucrose pulsing seems to suppress ethylene production by cut gentian flowers. Other studies have already indicated that postharvest sucrose treatment could delay ethylene production, for example, in cut carnation flowers (Dilley and Carpenter, 1975), sweet pea (Ichimura *et al.*, 1998) and sandersonia (Eason *et al.*, 2000).

In carnation, depletion of sugars in flowers was found to be accompanied by ethylene production (Nichols, 1966; Dimalla and Van Staden, 1980). In the present study, the overall trends of soluble sugar changes in the different pulsing treatments including the STS one seem to bear inverse relationship with ethylene production by cut gentian flowers. How soluble sugar content might be related to ethylene production is not clear. Nevertheless, it raises the interesting possibility that STS could inhibit ethylene production in petal cells via its capability to elevate soluble sugar content. Apart from the possible effect on ethylene production, an increase in soluble sugars within the petal could affect the pattern of gene expression giving rise to some gene products that are associated with vase life extension. This idea of sugar not just being a carbon and energy source but also specific signals in regulating plant gene expression has been studied in many non-floral systems (for example, Salanoubat and Belliard, 1989; Karrer and Rodriguez, 1992; Heim *et al.*, 1993; Godt *et al.*, 1995).

Sucrose pulsing delayed the onset of visual senescence in cut gentian flowers comparing to the water-pulsed samples. Sucrose-pulsed flowers have greater quantities of soluble and storage carbohydrates than control flowers. The senescence-associated reduction in soluble carbohydrate is delayed by sugar pulsing, and this was also reported in harvested sandersonia flowers (Eason *et al.*, 1997; Eason *et al.*, 2000).

In flower petals, the senescence process also consists of a loss of enzyme-mediated alterations of carbohydrates (Rubinstein, 2000). During senescence, the loss of sugars from gentian petals is not as rapid as that for asparagus spear tips (Eason *et al.*, 1996), it occurred at a very late stage of senescence which is very similar to that of sandersonia tepals (Eason *et al.*, 2000). The effect of sucrose solution for pulsing and the measurements of the accompanying changes in the levels of soluble sugars in the petals of cut gentian flowers are in general agreement with the contention that the maintenance of the carbohydrate pool in the petals is important for promoting flower longevity (Halevy and Mayak, 1979). The soluble sugars might contribute to the pool of respiratory substrates in the petal cells. It has been proposed that senescence is induced when the photosynthesis rate drops below a threshold (Dai *et al.*, 1999), although it is possible for photosynthetic rate to fall without loss of chlorophyll (Smart, 1994; Thomas and Howarth, 2000). When the hexose products of photosynthesis were consumed rapidly as a result of increased hexokinase activity, senescence was accelerated in both old and newly mature leaves (Dai *et al.*, 1999).

In the intact flower it can be postulated that its osmotic potential is maintained by import of carbohydrates, and presumably starch acts as a reserve and a buffer against an excessive osmotic potential, perhaps under hormonal control (Ho and Nichols, 1977). Osmotically equivalent solutions (i.e. 87.6 mM) of sucrose, fructose and glucose were equally effective as pulsing treatments of cut gentian flower stems in improving the vase life of the flowers. This finding is consistent with other studies showing that treatment of cut flower stems with a sucrose solution increased the levels of not only sucrose but also glucose and fructose within the petals (Shimamura *et al.*, 1997; Ichimura, 1998; Ichimura and Hisamatsu, 1999). It is possible that the

effectiveness of sucrose solution in delaying petal senescence might at least involve the 2 constituent sugars of sucrose, glucose and fructose. This suggestion is supported by aforementioned authors although Waithaka *et al.* (2001) found that the principal soluble carbohydrate in gladiolus florets was fructose, with substantially lower concentrations of glucose and sucrose. The suggestion is further supported by our findings showing the inability of a solution of sorbitol (87.6 mM), that is osmotically equivalent to the other 3 sugars, to extend the vase life of cut gentian flowers. It also follows from the finding with the osmotically equivalent sorbitol pulsing solution that the improvement in vase life of cut gentian flowers by sucrose, fructose or glucose pulsing solutions over the water control was not a result of initial osmotic shock or osmotic stress. On the other hand, it is conceivable that sorbitol is not a metabolisable sugar in gentian petal cells as plant cells generally have limited capacity to metabolise sugar alcohols (Thompson *et al.*, 1986). Interestingly, there was no increase in soluble sugar content in the petals following the sorbitol pulsing treatment. Also, the almost immediate decline in soluble sugar contents of both the water control and the sorbitol treatment correlates well with the shorter vase life of the treated flowers.

The data indicate that the pulsing solutions containing STS, sucrose, glucose and fructose were equally effective in promoting an increased level of soluble sugars in gentian petals. Ichimura and Hisamatsu (1999) found that glucose, fructose, sucrose and mannitol were detected in petals of snapdragon flowers after treatment with sucrose. In the present study, the onset of climacteric ethylene production was delayed by the sucrose treatment which suggest that the effects of sucrose for promoting bud opening and inhibiting flower senescence is attributed to an increase in sugar concentrations and the inhibition of ethylene synthesis. With the use of $^{14}C$ sucrose in the pulsing solution, it seems that much of the sucrose taken up during the pulsing treatment was not destined for or disposed of as a stored carbohydrate in the form of starch. A high level of radioactivity in the 80% ethanol-soluble fraction (presumably soluble sugar) was correlated with the elevated high level of soluble sugar in the petals pulsed with 87.6 mM sucrose and other carbohydrates and 0.5 mM STS.

The changes in the levels of starch in the gentian petals following the various pulsing treatments do not indicate a straightforward relationship with the changes in the soluble sugar content therein. In cut sweet pea flowers, starch level was higher in the sucrose-treated petals than in the water control (Ichimura *et al.*, 1998). This was not observed in the cut gentian flowers here. Besides, there are two particularly interesting points to note. First, the STS-pulsed petals had the least amount of starch throughout the vase-life experiment and yet they lasted longer than those in the other treatments did. Secondly, the sorbitol-treated petals had the most amount of starch at least in the initial few days of the vase-life experiment and yet they were the most short-lived ones among all the pulsing treatments. In the STS-pulsed petals, soluble sugar content was the highest among all treatments even the starch content was the least. This may contribute to the function that STS can somehow elevate α-amylase activity so that starch can be digested quickly to keep high soluble sugar concentration in the petal cells. While the sorbitol-treated petals had the highest level of starch but the lowest soluble sugar level among the treatments. It is possible that sorbitol, as a sugar alcohol, could not be metabolised by the petal cells, thus resulting in the shortest vase life (even shorter than water control samples).

Sugar pulsing may not have great influence on the cytosolic pool of sugars due to the cell's capacity to compartmentalise sugars to the vacuole. Therefore, although the data indicate that endogenous carbohydrate levels are greater in sucrose-pulsed tissue, the sugars may be sequestered into the vacuole so that they are not readily accessible to the rest of the cell (Eason *et al.*, 2000). It is meaningful if the subcellular levels of soluble carbohydrates could be measured for determination of which compartments contain elevated soluble sugars as a result of sugar pulsing.

### 4.2.2. *Protein content and enzyme activity changes in relation to flower senescence*

The senescence process also consists of a loss of proteins caused by activation of various proteinases (Rubinstein, 2000). Senescence is a genetically programmed process, the rate of which depends on the organ and species under consideration

(Thomas and Stoddart, 1980; Thomas and Smart, 1993; Buchanan-Wollaston, 1997; Thomas and Howarth, 2000). During senescence, N stored in mature leaves is remobilised for transport to younger or more demanding plant parts such as to reproductive organ e.g. flowers including petals and other reproductive parts. In the present study, total protein content was generally high at the blue bud stage and low in wilted flowers with large changes in petals compared to the other two parts, sepals and reproductive parts. Reproductive parts had the highest protein content at the blue bud stage and remained high till the flower wilted. This is consistent with reproductive parts being in a growing rather than senescence mode and a potential sink of mobilized nutrients from petals. Sepals showed the lowest protein content among the three parts and statistically there was no change throughout the three developmental stages, confirming that the sepal was not in the senescence mode.

The senescence of gentian petals seems to be associated with an apparent reduction in the protein content of the tissue. This finding is similar to those reported on carnation (Jones *et al.*, 1995), rose (Itzhaki *et al.*, 1998) and sandersonia (Eason *et al.*, 2000) but markedly different from that reported on chrysanthemum (Williams *et al.*, 1995) where the protein content of the petal tissue was only slightly reduced. Reduction of protein content in flower petals is recognized as a potential indicator of the flower longevity (Williams *et al.*, 1995). Therefore, the stability of the chrysanthemum proteins was considered to be associated with the relatively long post-harvest life of these potted flowers (25 days), while in gentian, a rapid decrease in protein content in the petals could well be associated with the relatively shorter post-harvest life (14 days). The reason behind this phenomenon may be due to the increased activity of a specific enzyme, e.g. endopeptidase or proteinase. This enzyme, similar to α-amylase, also becomes active during senescence (Smart *et al.*, 1995; Solomon *et al.*, 1999). Sucrose pulsing of sandersonia flowers delayed tepal senescence and altered amino acid metabolism (Eason *et al.*, 2000).

However, protein contents were found to be different among the petals treated with different pulsing solutions. Sucrose or STS treated petals had a much higher protein content than water treated petals did, particularly at the blue bud and open flower

stages. A study comparing α-amylase activity between water- and sucrose- or STS-treated gentian flowers indicated that α-amylase activities were significantly higher in sucrose- or STS-treated flowers than in water-treated control samples. Alpha-amylase activity decreased during senescence of the gentian flower. This result was consistent with that showing reduction in protein level during senescence of the gentian flower.

Not only protein content changed, but polypeptide profiles of the gentian flower extracts also changed in response to pulsing with sucrose or STS compared to the water control. SDS-PAGE analysis showed that extracts from sucrose- or STS-treated petals yielded one distinct polypeptide band of molecular masses of 24 kDa that was not seen in other extracts. This is of particular interest because it may be linked to increased longevity of the flowers after these pulsing treatments.

In general, the senescence of many petals is known to be accompanied by increased activities of several other hydrolytic enzymes which are thought to play a role in the degradation of macromolecules. Their actions allow remobilisation of nutrients from the petals to the developing ovary (Baumgartner *et al.*, 1975).

All flower parts at different stages showed amylase activity at different levels. In comparison with other floral organs such as sepals and reproductive parts (styles, stigmas, ovaries, filaments and anthers, etc.), the gentian petals had the highest amylase activity at the open flower stage. A high α-amylase activity in open flower petals might indicate that at this stage the petals needed more soluble sugars as energy sources / osmotic solutes to maintain the longevity of the petal cells. And this demand in turn might be linked to starch degradation quickly. It is not surprising that the amylase activity changes during flower development. In rose, amylase activity reached a peak in the petal 2 days after harvest and then declined gradually (Itzhaki *et al.* 1998).

In addition, changes in other enzyme activities, (for example, lipolytic acyl hydrolase) during petal senescence could be investigated. As it has been discussed above (4.2), the cell membrane deteriorates during petal senescence. De-esterification of

membrane lipids at the onset of senescence and the resultant release of free fatty acids can be attributed to lipolytic acyl hydrolase, an enzyme that has broad substrate specificity and can deacylate phospholipids directly (Galliard, 1980). Rose petals, for example, exhibit an increase in lipolytic acyl hydrolase at the onset of senescence that is accompanied by loss of membrane function (Borochov *et al.*, 1982). It is also an early and seminal feature of carnation petal senescence (Hong *et al.*, 2000). The action of lipolytic acyl hydrolase on membrane is a pivotal feature of senescence, setting in motion a cascade of events that leads to loss of cell function. The lipase gene encoding lipolytic acyl hydrolase in carnation petals that is strongly expressed coincident with the onset of petal senescence was isolated and characterised by Hong *et al.* (2000). The same enzyme in gentian petals could be a good candidate enzyme for further research.

### 4.2.3. *Molecular based changes during senescence of flower petals*

Senescence is an active process in which specific senescence-associated or senescence-enhanced gene products accumulate (Pontier *et al.*, 1999). These may play several potential roles leading to cell death: (1) Transcriptional systems could be generally or specifically inhibited, forcing the cell to shut down its most vital functions; (2) specific lytic enzymes could be induced to destroy individual components of cells; (3) energy resources could be severely compromised by synthesis of inhibitors or blockage of synthesis of critical components; (4) specific destruction of vital components with lethal consequences (Lockshin and Zakeri, 1991). The previous physiological and biochemical studies indicated that gentian flowers exhibited the substantial changes in the level of soluble sugar, starch, ethylene production, amylase and protein contents when the cut flower stems were treated with sucrose or STS compared to those treated with water. These might be brought about as a result of altered expression of ACC oxidase gene in response to the appropriate pulsing treatments of the gentian petals. More specifically in relation to the first aforementioned role, STS or sucrose pulsing might suppress the expression of ACC oxidase gene.

The objective of this part of study was to generate, by RT-PCR and standard PCR techniques, a fragment of the gentian petal ACC oxidase gene that can be used as a molecular probe to study the effect of STS or sucrose pulsing on ethylene production of gentian petals. As expected, a cDNA fragment of about 930 bp was generated with a set of primers designed to amplify the conserved region of ACC oxidase genes from several other plants. Optimized RT-PCR programme in this study led to the 931 bp putative ACC oxidase gene fragment which is very similar to that (954 bp) obtained from pigeon orchid using the same approach (Yang *et al.*, 1999). In this preliminary study of gene expression in gentian petals, RNA hybridisation was used to identify senescence-associated transcripts. RNA dot blotting analysis showed expression of the ACC oxidase gene in the petals of gentian, including high levels of expression in wilting petals. Analysis of the mRNA of normal carnation plants indicated the presence of ACC synthase mRNA and ACC oxidase mRNA in many tissues, but especially in senescing petals where there is a substantial peak of their mRNAs accumulation and their enzyme activity during the climacteric phase of petal senescence (Woodson *et al.*, 1992). The results of the present study are in agreement with this study. Gentian flowers exhibited the climacteric senescence (Zhang, 1996) and a high level of ACC oxidase mRNA from the senescing petals of the water-treated flowers was therefore be detected using the DIG-labelled ACC oxidase gene fragment. The sucrose- or STS-treated petals seemed to have less ACC oxidase mRNA in comparison with the water-treated petals. This indicated that the expression of ACC oxidase genes was, somehow, reduced to a lower level in the petals treated with sucrose or STS, while ethylene production seemed to be totally suppressed by the treatments. Hence, here some evidence has been obtained to suggest that sucrose, like STS, could act as an anti-ethylene agent at the molecular level, as far as ACC oxidase gene expression is concerned.

In the present study, the DNA was successfully labelled by non-radioactive DIG using a standard PCR technique with either Klenow enzyme or Taq DNA polymerase. A comparison of labelling efficiency between Klenow enzyme and Taq DNA polymerase was conducted and the results showed a difference between the two enzymes. The

effects of these two enzymes were clearly different with a much stronger band from Taq DNA polymerase and a very faint band from Klenow enzyme. Quantification of labelling efficiency of DIG-DNA by DNA dot blotting method also indicated the same difference between the two enzymes. The reason why Klenow enzyme did not work well comparing to Taq DNA polymerase in the reactions might be due to the long ACC oxidase gene fragments (931 bp) in gentian petals. Scharf and others (1986) indicated that the use of the Klenow enzyme worked well for the amplification of short fragments of DNA (200bp), but was disappointing for amplification of larger ones. Often the yields were low and the products showed size heterogeneity. This was probable due to the low annealing and extension temperature (37°C) that had to be used for the Klenow enzyme to be catalytically active (Scharf *et al.*, 1986). Taq DNA polymerase has an optimal extension rate (polymerization rate) of 35-100 nucleotides per second at 70-80°C, which is the optimum temperature range for the enzyme. Processivity, which is the average number of nucleotides incorporated before the enzyme dissociated from the DNA template, is relatively high for Taq DNA polymerase (Saiki *et al.*, 1988).

Many developmental processes of plants that lead to cell death, such as formation of tracheary elements (Fukuda, 1996) and senescence of flowers (Orzaez and Granell, 1997), are genetically programmed (Pennell and Lamb, 1997). It is recognised that nuclease activity increases during senescence, resulting in DNA cleavage and degradation (Gan and Amasino, 1997; Yan and Yang, 1998). Concomittant with this process, expressin of various senescence-associated genes have been shown (Buchanan-Wollaston, 1997, Can and Amasino, 1998). Those genes identified as senescence-enhanced include proteases, RNAase, lipases and some enzymes of N metabolism, as well as the housekeeping genes which continue to be expressed during senescence as well as in non-senescent tissues (Thomas *et al.*, 1992; Smart, 1994; Buchanan-Wollaston, 1997; Quirino *et al.*, 1999). However, questions about the regulation of programmed cell death during senescence have remained unanswered. Thus, none of the senescence-associated genes identified up to now appears to be a regulatory gene that has a function in initiation or control of senescence (Buchanan-Wollaston, 1997).

# Chapter 5. General discussion and Conclusions

The flowering season of gentians grown in the nursery is very restricted, being from early January to middle of March each year in New Zealand. Growing gentians in greenhouse was impracticable due to the nature of the plants. To supply enough fresh flower materials for research from time to time in a year, the idea of generating *in vitro* flowers of gentian was successfully explored here. The success depended largely on setting up a controlled rather than an unreliable spontaneous *in vitro* flowering system. The results are very exhilarating, meaningful and of great help for further studies. We regard this as a very basic and important aspect of study in the work presented here. Otherwise, it is impossible to carry on so much physiological, biochemical and molecular work as what we have described in this study.

The present study indicated that the *in vitro* flowering of *G. triflora* is affected by shoot position, sucrose concentration, cytokinin concentration and pH value in light conditions. $GA_3$ had no effect on improving *in vitro* flowering of this flower crop. Shoot and flower formation differs in their requirement for PPFD. Shoot production is evidently dependent upon a low PPFD (30 $\mu mol\ m^{-2}\ s^{-1}$) whereas flower production is greatest at moderate PPFD (60 $\mu mol\ m^{-2}\ s^{-1}$). The *in vitro* flowering system as presented in this study suggested that the culture of *G. triflora* as a technique for investigation of flowering is promising. Compared to the intact plant systems commonly used in studies of flowering, the *in vitro* flowering system offers an attractive alternative. Except the flower size (corolla length), pollen germination and pollination rate, *in vitro* flowers are largely similar to *in vivo* flowers in other aspects, such as the shape and colour of the flowers and petals, the number of anthers, the percentages of fully developed stigma, the shape, size and colour of pollen grains, the

viability of pollen, etc. This provided evidence for *in vitro* flowers as an alternative in the proposed study on gentian flowers. Furthermore, the flowers can be obtained from more than 80% of the *in vitro*-cultured nodal explants under defined aseptic culture conditions within 9 – 12 weeks. By repeating several cycles of this *in vitro* flowering from sub-culturing the nodal explants, flowers can be obtained throughout the year for experimental studies including vaselife treatments. The application of *in vitro* flower for vaselife treatment and studies is novel. Also, with further experiments, this should lead to a better understanding of the reproductive biology of gentians.

Pulsing with 87.6 mM sucrose solution clearly improved bud opening and flower vase life in cut gentian (*Gentiana triflora*). Total soluble sugar concentration in the petals increased markedly by the sucrose treatment. Comparatively, 0.5 mM STS pulsing treatment has similar effect with 87.6 mM sucrose pulsing treatment in improving colour changes of the white buds, blue buds opening and longevity of open flowers. These results suggested that the effectiveness of sucrose in promoting bud opening and inhibiting flower senescence could be ascribed to the increase of the sugar concentration in the flowers. Sucrose uptake during pulsing treatments was confirmed using radioactive $^{14}$C-sucrose. The result further suggested that soluble sugar might play an important role in the longevity of *G. triflora* petal.

The STS-pulsed petals had the least amount of starch throughout the vase-life experiment and yet they lasted longer than those in the other treatments did. While in the sorbitol-treated petals had the most amount of starch at least in the initial few days of the vase-life experiment and yet they were the most short-lived ones among all the pulsing treatments. It would be interesting to develop strategies, for example, transgenic flower, that have modified biochemical mechanisms in reducing / elevating starch content in the petals for an examination of petal longevity.

Gas chromatograph (GC) analysis revealed that ethylene production of the flowers was suppressed by the sucrose or STS treatment. In the present study, the overall trends of soluble sugar changes in the different pulsing treatments including that of STS seem to bear inverse relationship with ethylene production by cut gentian flowers. These

results strongly suggest that sucrose or STS pulsing solution has anti-ethylene effect, which is likely to contribute to its effectiveness in extending the vase life of cut gentian flowers. The results of GC analysis showed that the time of ethylene started to accumulate (4 days after pulsing treatment) was the time the starch started to decline (4 days after a pulsing treatment). This seems to reveal a relationship between starch decreasing and the increase of ethylene production in the petals. It raises the interesting possibility that sucrose or STS could inhibit ethylene production in petal cells via its capability to elevate soluble sugar content while decrease starch content. From this, we proposed that an increase in soluble sugars within the petals could affect the pattern of gene expression giving rise to some gene products that are associated with vase life extension. This idea of sugar not just being a carbon and energy source but also specific signals in regulating plant gene expression has been studied in many non-floral systems (for example, Salanoubat and Belliard, 1989; Karrer and Rodriguez, 1992; Heim *et al.*, 1993; Godt *et al.*, 1995). There is growing evidence that in many cases ethylene exposure results in autocatalytic ethylene production which led to altered gene expression (Woodson and Lawton, 1988; Broglie *et al.*, 1986). The results presented in this study indicated that exogenous ethylene could induce autocatalytic ethylene production in gentian petals and result in shortened flower vase life. Although autocatalytic ethylene production was shown, it remained to be investigated more closely if altered gene expression in gentian petals would follow.

In our previous study, it has been clear that when treated with sucrose, the soluble sugar content increased while starch content decreased in gentian petals. To this, we postulated that it could be amylase which played a role in the hydrolysis of starch in gentian flower petals. We, therefore, would like to examine a bit more closely to see how starch could be hydrolyzed in the petals. Four experimental approaches were conducted: (1) Total amylase assay from freshly collected *in vitro* flowers at different developmental stages; (2) Determination of the type of amylase; (3) Determination and quantification of α-amylase activities from gentian flowers pulsed by selected solutions, and (4) Paper chromatography of the amylase reaction products. The results clearly confirmed the presence of mainly α-amylase activity in the crude extracts of the petals and other flower parts tested.

A study on comparison of α-amylase activity between water- and sucrose- or STS-treated gentian flowers indicated that α-amylase activities were significantly higher in sucrose- or STS-treated flowers than in water-treated control samples. This indicates that sucrose or STS at concentration of 3% (w/v) or 0.5 mM, respectively, could increase α-amylase activities in gentian flowers, which presumably contributed to a decrease in the starch content in the petal treated by sucrose or STS.

Studies on protein content indicated that gentian flower senescence was associated with reduction of the protein content in the petals as well as a loss in their fresh weight. Sucrose or STS pulsing treatment could increase protein content in the petals in comparison to that of water treated petals. The sepals displayed different behaviours compared to the petals because there were no changes in fresh weight and protein content during the course of flower development and no response to the pulsing treatment with sucrose or STS. It is likely that the sepals may produce no or little ethylene. This remains to be investigated.

Another very interesting phenomenon is that new polypeptides of low molecular weights are detected on SDS-PAGE gels used for analysis of the protein profiles of the petals treated with sucrose or STS. This suggests the possible changes in gene expression resulting from the pulsing treatments. Further characterization of the new polypeptides and their relationship to flower longevity will need to be studied, perhaps using 2-D gel electrophoresis and N-terminal amino acid sequencing to probably reveal their identities.

Traditionally, the ideas on senescence mechanisms fall into two major groupings, nutrient deficiencies (e.g., starvation) and genetic programming (i.e., senescence-promoting and senescence-inhibiting genes). Considerable evidence indicates that nutrient deficiencies are not central senescence program components, while increasing evidence supports genetic programming (Noodén *et al.*, 1997). It is assumed that during senescence of gentian flowers, the rate of ethylene production increased and this parallels with the rapid increase in the activity of ACC oxidase, a key enzyme in the biosynthetic pathway of ethylene formation. Here, we presented dot blot data to

suggest that ACC oxidase gene expression could be suppressed by the administration of sucrose or STS. Certainly, these pulsing treatments were largely effective in halting ethylene production in gentian petals.

In conclusion, we would like to recommend that *in vitro* flowers are suitable materials for vaselife studies, which can only benefit from enough fresh flowering materials year round. The petal senescence in gentian is an active process involving many physiological and biochemical changes, and includes the expression of ACC oxidase gene. The process appears to be regulated by ethylene. The sucrose- or STS-suppressed-ACC oxidase gene seems to have succeeded in suppressing the level of ACC oxidase mRNA. This, in turn, is likely linked to reduced ethylene production and extension of gentian flower vase life. Whether the reduction of mRNA is the result of decreased transcription or changes in mRNA processing is not known at the present time. However, our results suggested that changes in petal physiology in response to sucrose or STS were associated with rapid alterations in gene expression. Both sucrose and STS could be inhibitors in ethylene biosynthesis through regulating ACC oxidase gene expression.

In the next few years, if the cDNA fragments obtained in this study using RT-PCR technique could be employed to isolate a full-length cDNA clone of gentian ACC oxidase gene, transgenic gentian plants would then be generated. The generation of this transgenic gentian plants with antisense ACC oxidase cDNA constructs enables a study to see if the ability to synthesize ethylene has been reduced or eliminated and if vaselife could be improved. This would hopefully reflect similar findings in transgenic carnation and tomato and reinforce the concept of improving properties, such as postharvest qualities, by genetic engineering rather than by long-term classical breeding or the application of chemicals.

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# Appendixes

## *Appendix A Buffers for protein and enzyme assay*

### *A1 0.1 M Sodium phosphate buffer*

| pH | Volume of 0.1 M $KH_2PO_4$ (ml) | Volume of 0.1 M $Na_2HPO_4 \cdot 2H_2O$ (ml) |
|---|---|---|
| 4.0 | 10.0 | 0 |
| 5.0 | 9.75 | 0.25 |
| 6.0 | 9.0 | 1.0 |
| 7.0 | 4.0 | 6.0 |
| 8.0 | 0.5 | 9.5 |

### *A2 0.2 M Acetate buffer*

| pH | Volume of 0.2 M acetic acid (ml) | Volume of 0.2 M sodium acetate (ml) |
|---|---|---|
| 5.2 | 10.5 | 39.5 |

### *A3 Iodine reagent*

To make 10 ml iodine reagent, mix 0.1 ml iodine stock solution with 9.9 ml $dH_2O$

### *A4 Iodine stock solution*

Mix 0.06g iodine and 0.60g potassium iodine with 10 ml of $dH_2O$. Stored in a dark bottle.

### *A5 0.1 M citric buffer*

| pH | Volume of 0.1 M citric acid (ml) | Volume of 0.1 M Sodium citrate (ml) |
|---|---|---|
| 3.0 | 46.5 | 3.5 |

### *A6 5 × SDS-PAGE sample buffer*

4 ml $dH_2O$; 1.0 ml of 0.5 M Tris-HCl, pH 6.8; 0.8 ml Glycerol; 1.6 ml 10% (w/v) SDS; 0.4 ml 2-mercaptoethanol; 0.2 ml 0.05% (w/v) bromophenol blue (SDS reducing buffer, store at room temperature).

### *A7 0.2 M Sodium succinate buffer*

Mix 25 ml of 0.2 M succinic acid (32.6 g / 1000 ml $H_2O$) with 37.5 ml of 0.2 M NaOH then make up to 100 ml by $H_2O$ (pH 5.5).

### *A8 Solution A*

30% (w/v) acrylamide plus 1% (w/v) bis-acrylamide

### *A9 2 × sample buffer (5 ml)*

Mix 40 µl Pharmalyte 2D (pH 3-10), 160 µl Resolyte (pH 4-8) and 3 ml Glycerol with 1.8 ml $dH_2O$

## Appendix B Buffers for Native PAGE gel electrophoresis

### *B1 Separating gel solution (75 ml for 2 gels)*

30 ml Acrylamide stock (29.2 % acrylamide and 0.8% Bis); 26.5 ml $ddH_2O$; 18.75 ml separating gel buffer (1.5 M Tris-HCl, pH 8.8, 0.4% SDS); 45 µl TEMED; 450 µl of 10% ammonium persulfate

### *B2 Stacking gel solution (30 ml for 2 gels)*

4.8 ml Acrylamide stock (29.8% acrylamide and 0.8% Bis); 17.7 ml $ddH_2O$; 7.5 ml separating gel buffer (0.5 M Tris-HCl, pH 6.8, 0.4% SDS); 30 µl TEMED; 75 µl of 10% ammonium persulfate

### *B3 Electrode buffer*

Dissolve 57.6 g glycine; 12 g Tris base; 2 g SDS and 0.4 g sodium azide (lower buffer only) into 2 liter $dH_2O$

# Appendix C Solutions used in Molecular work

## *C1 RNase-free water*

Diethyl pyrocarbonate (DEPC)-treated water is prepared by adding 1 ml DEPC to 1 litter of double-distilled water (0.1% DEPC v/v) and stirring overnight at 37°C. The DEPC-treated water is then inactivated by autoclaving at 20 psi for 20 min.

## *C2 TAE (Tris-Acetate) Buffer stock solution (50X)*

Per litter:

| | |
|---|---|
| Tris base | 242 g |
| Acetic acid glacial (glacial acetic acid) | 57.1 ml |
| 0.5 M EDTA (pH 8.0) | 100 ml |

## *C3 DNA Molecular Weight Marker (Hind III digest of λ-DNA)*

| | | |
|---|---|---|
| (1) λ-DNA digest stock (0.25μg / μl) | 20 μl (5μg ) | |
| (2) 50 × TAE buffer | 1.4 μl | |
| (3) DNA loading buffer (Type III) | 11.7μl | |
| (4) Sterile $dH_2O$ | 36.9μl | |
| Total Vol: | 70 μl | Store at – 20°C |

## *C4 DNA loading buffer (Type III)*

0.25% bromopheno blue, 0.25% xylene cyanol and 30% glycerol in $H_2O$

## *C5 Electrophoresis buffer (1×TAE buffer)*

To make 250 ml:

Mix 5 ml TAE stock solution with 245 ml $dH_2O$

## *C6 Blocking solution (1×)*

1% (w/v) blocking reagent (Boehringer Mannheim) in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5)

### *C7 Antibody solution*

Dilute the Anti-Digoxigenin-AP (Boehringer Mannheim, 750 units/ml) 1:5000 in blocking solution

### *C8 Washing buffer*

maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 3% (v/v) Tween 20

### *C9 Detection buffer*

100 mM Tris-HCl, 100 mM NaCl, pH 9.5

### *C10 Colour substrate solution*

Add 100 µl of NBT/BCIP solution (Boehringer Mannheim) to 5 ml of detection buffer

### *C11 RNA dilution buffer*

Make following proportion DEPC-treated dd$H_2O$ : 20x SSC (3 M NaCl, 0.3 M sodium citrate at pH 7.0) : formaldehyde = 5:3:2

### *C12 Pre-hybridization solution (high SDS hybridization buffer)*

7% SDS, 50% formamide (deionized), 5x SSC (0.75 M NaCl, 75 mM sodium citrate, pH 7.0), 2% blocking reagent (Boehringer Mannheim), 50 mM sodium-phosphate (pH 7.0), and 1% N-lauroylsarcosine

### *C13 Washing solution (2×)*

2 × SSC (0.3 M NaCl, 30 mM sodium citrate, pH 7.0) containing 0.1% SDS

### *C14 Washing solution (0.1×)*

0.1 × SSC (15 M NaCl, 1.5 mM sodium citrate, pH 7.0) containing 0.1% SDS

### *C15 Washing buffer*

maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5), 3%(v/v) Tween 20

# Appendix D Programs for running PCR

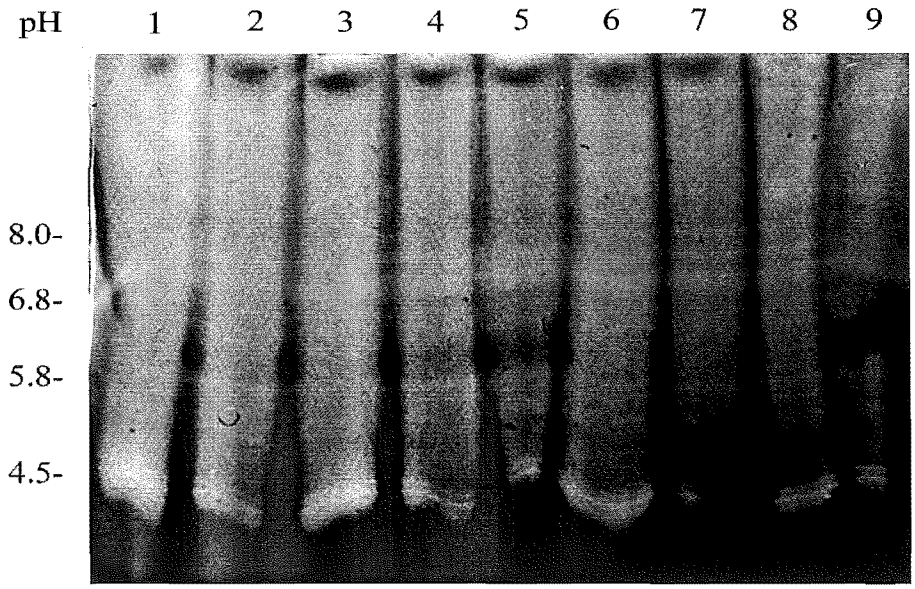
## *D1 Programme TED I*

This was set specifically for running RT-PCR when using primers AO1 and AO2: The temperature profile was set as following steps: (1) equilibration at 50°C for 30 min; (2) denature template at 94°C for 2 min; (3) denature template at 94 for 30 s; (4) annealing at 60°C for 30s; (5) elongation at 68°C for 2min; (6) the cycle number was 40 from step 3 to step 5. It took about 7 hours to complete the reaction.

## *D2 Programme TED II*

This was set for running PCR particularly for amplification of DNA: (1) heating at 94°C for 2 min to denature the first strand products; (2) denaturing at 94°C for 1 min; (3) annealing at 60°C for 45s; (4) extending the DNA at 72°C for 2 min; (5) carrying 35 cycles from step 2 to 4; (6) end.

## Appendix E Isoelectric focusing




**Isoelectric focusing of $\alpha$-amylase isozymes from parts of open *G. triflora* flowers after 24 h pulsing with water, 3% sucrose or 0.5 mM STS**

Lanes 1-3: extract of petal from flowers pulsed with $H_2O$, sucrose or STS, respectively; lanes 4-6: extract of sepal from flowers pulsed with $H_2O$, sucrose or STS, respectively; lanes 7-9: extract of reproductive parts from flowers pulsed $H_2O$, sucrose or STS, respectively. 15 μl enzyme was loaded in each lane on the pH gradient gel, focused and stained for amylase activity with iodine reagent.

# Appendix F Concentrations of DIG labelled DNA

## F1 Concentration of DIG labelled DNA by Klenow enzyme

| Dilution steps | Stepwise dilution in DNA dilution buffer | Total dilution | Final concentration |
|---|---|---|---|
| (a) | 5 µl / 45 µl | 1:1 | 1 pg / µl |
| (b) | 5 µl / 45 µl | 1:10 | 0.1 pg / µl |
| (c) | 5 µl / 45 µl | 1:100 | 0.01 pg / µl |
| (d) | 5 µl / 45 µl | 1:1000 | 0.001 pg / µl |
| (e) | 5 µl / 45 µl | 1:10000 | 0.0001 pg / µl |

## F2 Concentration of DIG labelled DNA by Taq polymerase

| Dilution steps | Stepwise dilution in DNA dilution buffer | Total dilution | Final concentration |
|---|---|---|---|
| (a) | 5 µl / 45 µl | 1:10 | 10 pg / µl |
| (b) | 5 µl / 45 µl | 1:100 | 1 pg / µl |
| (c) | 5 µl / 45 µl | 1:1000 | 0.1 pg / µl |
| (d) | 5 µl / 45 µl | 1:10000 | 0.01 pg / µl |
| (e) | 5 µl / 45 µl | 1:100000 | 0.001 pg / µl |